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What future for European fast reactors?

Of all the neglected technologies of the past few years, the technology of the fast reactor is the most pathetic. Long gone are the days when governments in the industrialized world were declaring their intention of running up one or two demonstration plants confident in the hope that their electricity utilities would then be able to make limitless (and cheap) electricity without much trouble. As the years have gone by, governments have become more and more hesitant, even pusillanimous. In the United States, fast reactor development is in the doldrums — the demonstration reactor at Clinch River consists largely of a collection of components not yet put together, while all but a few of the small-scale reactors being built or operated ten years ago have now bitten the dust. The trouble at Clinch River is partly financial (the cost of completing the reactor would now be far greater than the original estimate), partly technical (the design leaves much to be desired) and partly political — fast reactors are regarded by the US Administration as the doorway to the “plutonium economy” and the rapid proliferation of nuclear weapons to lie beyond that, while fast reactors have become prime targets for those who go about with “No nukes” on their bumper stickers or tee-shirts.

Outside the United States, the desuetude is less conspicuous even though the brash optimism of the 1950s has largely evaporated. There are substantial prototype fast reactors in France, the Soviet Union, the United Kingdom and West Germany, while a reactor capable of generating 1,200 MW of electricity (and called Super-Phénix) is being built in France with financial support from West Germany and, to a lesser extent, Italy. As the months go by, the prospects improve that, by 1983, this reactor will turn out to be the first full-scale reactor of its kind. The French are already talking of the reductions of capital costs that will come about when the sixth or seventh replicas of Super-Phénix are built. Now, it seems, this talk has wooed the UK Atomic Energy Authority away from its long-standing belief in its own supremacy. This, at least, is one reading of Sir John Hill's welcome last week for the proposal that the United Kingdom might join the European collaboration concerned with Super-Phénix (*Nature*, 18 September). The decision now rests with the British government but will also, no doubt, be influenced by the course of the negotiations on commercial terms which are still to be completed. How sensible is this course of action? What, in any case, are the prospects for fast reactor technology? And does it follow that the nuclear business, in France certainly but perhaps in the rest of Europe, is embarked on what many of its critics consider to be a dangerous path?

The proposal that British interests in fast reactor technology should be met within the framework of some wider collaboration is probably not so much a choice as a Hobson's choice. For several years, it has been obvious that no amount of successful operation of the Prototype Fast Reactor at Dounreay would bring into being the heavy industry that would be needed if fast reactors were ever to be built in earnest. The National Nuclear Corporation (now to be reorganized again, but probably to be made more effective in the process) is having to go through all kinds of gyrations to take on the design and construction of a single pressurized water station on which construction cannot begin for another two years. At the same time, to create the capacity for building fast reactors, especially when the demand for these is entirely unknown, would be impossible. Such an attempt would be a folly. On strictly practical grounds, it is prudent to look for some international arrangement under which the labour of building fast reactors

could be divided, together with the profits (if any) in doing so. Wider considerations, European collaboration and all that, reinforce the point. So far, so good.

As always, there are several snags. Is Super-Phénix such a mature fast-reactor design that it will be possible, as the French hope, to use it as the model on which to fashion a whole generation of replicas? Or will it be necessary, after 1983, to build yet another demonstration plant, and to incur the substantial extra costs of doing so? Answers to these questions (which cannot be simple) will no doubt help to determine the British government's response to the Atomic Energy Authority's request. The choice, it will be remarked, is again not an open choice between collaboration and going it alone. Given the government's commitment to a public inquiry before a commercial fast reactor could be built, the Atomic Energy Authority would have to kick its heels for a further five years before the construction of a commercial fast reactor could begin. While scratching their heads over these alarming questions, ministers and the management of the AEA should reproach themselves (and/or their predecessors) for having, aloof and toffee-nosed, scorned the proposals for European collaboration on the development of fast reactors in the decade following the late 1950s.

But why the hurry? And what, in any case, is the objective? If, in the United States, nuclear policy is based (as it has been for the past three years) on the principle that the recycling of nuclear fuel is economically unnecessary, why should European governments be rushing ahead? The French position is clear and characteristic. Self-sufficiency, or near self-sufficiency, is in itself a legitimate goal. The Commissariat à l'Energie Atomique has calculated that it might be possible to generate a substantial proportion of French electricity production from indigenous uranium, but only with the help of breeder reactors extracting fission energy from uranium-238 (via plutonium-239) as well as from uranium-235. This will not, of course, prevent the French from making deals with Australia for the supply of uranium ore (like that announced last week) — that will be a sensible way of eking out supplies. Elsewhere in Europe, self-sufficiency is less obviously a part of public policy, although few European can take much comfort from American assurances that uranium prices will be relatively low (less than £35 per kilogram of U_3O_8) for the remainder of this century. Prices could leap up, as they have done on several occasions in the past, if (or rather when) there is a revival of confidence in nuclear power in the United States. In most European countries, however, there is no immediate need for a fast-reactor programme on the scale now foreseen in France. The most urgent need is that potential users should become so familiar with this new technology that they will be able to handle it confidently. Much also needs to be learned about the economics of fast reactors, and of the fuel cycles on which they are based. The case for pushing ahead quickly with fast reactor technology is that its long-term importance will be demonstrated only when a number of reactors have been used commercially, by European utilities of all kinds.

If this objective were more clearly recognized, some of the present reactions against the building of fast reactors would, or should, be less intemperate than they are. It must, however, be expected that others will persist. Fears for the safety of fast reactors are rife, but, as commonly stated, are often misplaced. It is in everybody's interests that such anxieties as there are should be widely understood. The fact that a core of a fast reactor



contains more than enough fissile material to cause a nuclear explosion does not make every fast reactor a potential bomb.

The most likely sources of potential hazard are, on the contrary, mundane — the apparently devilish capacity of liquid sodium to work its way through leaks and cracks, the formation of vapour bubbles in the circulating sodium which can increase the reactivity of the system (at least if it is physically large enough) and the possibility of chemical interaction between liquid sodium and fuel cans or impurities (such as water). The most likely source of criticality problems is not the formation of a single critical mass of fissile material but, rather, mechanical oscillation within the reactor core which, on paper at least, may be symmetrical enough to set in train a sustained and rapid increase of reactor power. The fact that the nuclear reaction involves fast (1 MeV or so) neutrons rather than thermal neutrons is not in itself a source of potential hazard. The power density of the system (400 W per cm³ in Super-Phénix) does, however, carry heat-transfer engineering into a novel field, while the use of liquid sodium as a heat transfer medium presents another set of once unfamiliar problems. On balance, however, there is no reason to dispute the claim of the nuclear engineers that individual fast reactors need be no more hazardous than conventional reactors. In Britain, the intended public inquiry, unfortunately now delayed at least until 1985, will provide sceptics a splendid opportunity to test the proposition.

Fears that fast reactors mean a greater risk of proliferation are less substantial, and should now be exorcized. The argument is

that if plutonium is separated from spent fuel from thermal reactors, stocks of plutonium will increase as will the opportunities for theft and illicit diversion into bomb-making. In its simplest form, the argument perversely overlooks the simple truth that reprocessing facilities are at present few and far between. The bomb-making states, anxious as they are that the nuclear club should not be enlarged, control the only substantial plants now in operation. Others, even that being built in Brazil, are under international control. India is the exception that proves the rule — its reprocessing facilities are uncontrolled, but their capacity is small. In the past year, and especially in the wake of the International Fuel Cycle Evaluation, there has been much talk of internationally operated reprocessing facilities.

Such schemes, imaginative though they are, are not, however, the most urgent needs in the international effort to control proliferation. Certainly the pace of fast reactor development now on the cards is not going to increase the opportunities of governments to join the nuclear club, at least in the remainder of this century. Moreover, given that such plutonium as fast reactor programmes will produce will accumulate in the stockpiles of well-ordered states (and will also, not doubt, be put back into fast reactors as quickly as possible) there is no reason to think that the chances of illicit diversion will be proportional to the total quantity of plutonium in existence. Safety, in brief, is something to worry about. The risk of proliferation at least for the time being, is not.

Technical innocence in the public service

With much of the British Scientific Civil Service still seething over the pay settlement for this year (*Nature*, 24 July), the Civil Service Commission on Tuesday went some way to acknowledging that scientists in the Civil Service often have a raw deal. The publication of the *Review of the Scientific Civil Service (1980)* (Cmnd 8032, HMSO, £6.10), prepared by a group of officials with Dr Martin Holdgate, Chief Scientist at the Department of the Environment, is not pure emollient, however. The remedies which the review suggests, valuable as far as they go, do not touch the problem which many people (not only scientists in the Civil Service) consider the most serious — the continued separation of scientists (and of other professionals) from the Administrative Group, the body chiefly responsible for the formulation of policy within the British government and within which true mandarins grow up. Although, at the higher reaches of the Scientific Civil Service, a handful of posts are considered as "open" grades, it is inevitable that for most people in the public service the Holdgate review will seem to rub salt in open wounds. So too will the date in parentheses at the end of the title, which is an awkward reminder of how often the question has been considered, and to how little effect.

The case for a merger of scientists (and others) in the public service with the administrators is not to feather the nests of scientists, nor to ensure that each and every one of them can look forward, from the beginning of his career, to being a powerful head of a Department of State, with a briefcase and chauffeur to match. Many scientists would in any case prefer to avoid that path. The need is rather that the public service as a whole should be more able to understand the problems of a technical character with which it has increasingly to battle. The Holdgate review acknowledges this need at the outset of its report. It goes on to record the failure, in the past few years, of the schemes devised to help scientists transfer from their ghetto to the Administrative Group. The explanation is, of course, straightforward. Scientists in early or mid-career do not relish giving up their science to become administrators. Afterwards, it is often too late. The Holdgate remedy is that, in planning people's careers in the Scientific Civil Service, care should be taken that promising people should be given training in the "management skills" supposed essential to administration. The trouble is that without a common understanding throughout the public service that posts

as administrators would be found for those wishing to transfer, more training may simply mean more frustration. In any case, for as long as most of those responsible for policy-making persist in their innocence of technical questions, the public service will be less effective than it needs to be. This broad question is outside the terms of reference of the Holdgate review, for reasons which are not necessarily sinister. Nobody should forget that it is the question most in need of answering.

If tinkering with the present system can help, however, Holdgate and his men (*sic*) have some useful things to say, especially on the careers of scientists in the public service. Scientists need to know more clearly than at present what their career prospects are, and to be helped (if they wish) to broaden them. Merit needs to be rewarded, especially in those following less glamorous careers such as the provision of technical services. Decorously, the report skirts round the issue of pay, and also timorously accepts that probationary and fixed-term appointments to the Scientific Civil Service would be impracticable. The truth is that they would be unwelcome, which is another matter. The review comes close to acknowledging the need for a more effective scientific contribution to general policy with its plea (to the administrators) that they should more openly look for and welcome scientific advice within the public service. We shall no doubt see (or not see) what happens.

What will happen now? The Civil Service Department (responsible for employing all Civil Servants) will urge everybody in sight to follow the Holdgate recommendations. After a few months, or a few years, this review will join its predecessors in the archives of British public administration. The innocence of the Civil Service as a whole of what the modern world is like will be found to persist. It will be forgotten, then, that the Holdgate review included within its terms of reference the question of what the Scientific Civil Service is for; its report dutifully includes a list of the duties scientists in the public service perform. Now and again there is a hint that the committee of officials knew it was avoiding a crucial issue — and the recommendation that research establishments might be decentralized may absolve it of some blame. But is it not high time that somebody asked seriously how best 17,000 of the most highly qualified scientists in Britain should serve the public interest? Influencing policy is one thing. Making government research establishments more useful is another.

US woos African states with science

Washington

Trade, it seems, no longer follows the flag but the lab. Washington's top science administrators departed for Africa last week for a ten-day trip aimed at stimulating scientific and technical cooperation with four "moderate" African states — Nigeria, Kenya, Senegal and Zimbabwe.

The sixteen-person delegation is headed by Dr Frank Press, director of the Office of Science and Technology Policy (OSTP). Other members include the administrator of the National Aeronautics and Space Administration, the acting director of the National Science Foundation and the director of the National Institutes of Health.

The practical goal of the delegation is to sign a number of agreements with the four countries for programmes of mutual cooperation. These range from setting up an experimental rural telecommunications system based on satellite technology in Senegal to a broad programme of vocational training courses for Nigerian science and engineering students in US universities and technical colleges.

The delegation's visit is also part of a broader strategy to strengthen links with four African countries with which the United States currently has the best relations. The intention is to help these countries to gain economic stability by promoting their technical efforts — and this in turn should provide a basis for their political stability.

In at least one case there are pragmatic reasons for the United States to want to maintain close political links. Nigeria is at present the nation's second largest supplier of petroleum (after Saudi Arabia); and continuation of this supply is important for the nation's security.

Some money is to be made available by the federal government so that agreements reached during the visit can be followed through, for example by arranging for the reciprocal visit of African scientists and policy makers to the United States. But for many countries, additional fiscal resources are less important than access to people and institutions in the United States able to help with their development problems.

OSTP expects agreements for cooperation to be signed in fields such as marine sciences, environmental resources, agriculture, remote sensing, satellite communications, alternative energy sources and the management of scientific research and technical information.

Three of the countries — Senegal, Kenya and Zimbabwe — will be able to benefit from financial and technical assistance offered under the term of US aid policies. In the fourth, Nigeria, the average income is above the limit for concessional terms to be permitted, and the United States will be looking for costs to be reimbursed.

Many of the agreements due to be signed cover areas that would have been the responsibility of the ill-fated Institute for Scientific and Technological Cooperation (ISTC). The setting up of ISTC was proposed by President Carter in 1978 and authorized by Congress last year, but because of a dispute with the Senate over funding is unlikely to come into existence. However, Dr Press said that the scope of the cooperative agreements, which will include feasibility studies of a Nigerian-based tuna industry and a project on ammonia-based synthetic fuels in Zimbabwe, will go beyond the role that had been intended for ISTC. The mission, according to Dr Press, has the full and active backing of President Carter, who had for example discussed it with Dr Robert Mugabe, Prime Minister of Zimbabwe, who

visited Washington last month.

One disappointment for US — and possibly African — officials is that the delegation will not include Dr John Slaughter, a black who has been nominated by President Carter as director of the National Science Foundation.

After an inter-party dispute in the Senate over whether Republican members should assent to the appointment of Democrat nominees in the last few months before the Presidential election, Dr Slaughter's nomination was approved by a Senate committee last Wednesday, and is expected to move swiftly through the full Senate with little opposition. The delay has meant that Dr Slaughter will not be on the delegation and his place will be taken by Dr Donald Langenberg, the foundation's acting director.

David Dickson

Still looser UK guidelines

The Genetic Manipulation Advisory Group (GMAG), responsible in the United Kingdom for the administration of guidelines on recombinant DNA research, has taken a substantial step towards oblivion. From now on, according to the latest revision of its guidelines applicable to university and industrial laboratories, experiments planned for the least stringent of the four categories of safeguards will not be scrutinized in advance by GMAG. Instead, the laboratory safety committee will have to carry through the standard GMAG hazard assessment and assure itself that proposed experiments do indeed belong in Category I. Laboratories will be required to inform GMAG in September each year of all experiments carried out under this licence in the previous year.

Sir William Henderson, chairman of GMAG, estimated last week that this step will relieve GMAG of 90 per cent of its present work. Because of rules which allow much other research to proceed once GMAG has been notified, it is estimated that only some two per cent of the experiments now being planned will have to await formal permission from GMAG.

The proposal that laboratories should be granted an annual licence to carry out experiments in Category I was first considered by GMAG in November 1978, at its first radical revision of the guidelines. The committee then considered that further information was necessary before the matter could be decided.

Sir William and other members of the committee are at pains to emphasize that this most recent revision of the GMAG procedures has been made possible by the accumulation of information in the past two years, and especially by evidence that the capacity of genetically manipulated

bacterial cells to produce foreign protein molecules is limited. The assumption is that the production of foreign protein molecules from a single *Escherichia coli* bacterium with a foreign gene at a plasmid site chosen for expression will not exceed 10^6 . This, the argument goes, provides ways of quantifying the maximum amount of foreign protein produced in the infection of, say, the human gut by genetically altered bacteria. On this basis, the maximum amount of insulin produced in the gut by *E. coli* carrying an insulin gene could not exceed the equivalent of 0.6 units of the hormone, compared with the 20 or 30 units normally circulating in the body.

Laboratory safety committees will be expected to verify such estimates made by those wishing to carry out experiments, and to adjudicate specific proposals for categorization of the experiments concerned. GMAG itself expects that there will be further revisions of the guidelines, no doubt tantamount to further relaxations, in the not too distant future.

GMAG's own concerns are increasingly with proposals for large-scale manufacture — although the volume of applications so far has, apparently, been surprisingly small. Sir William considers, however, that both in respect of what GMAG requires from industrial applicants and more generally, British regulation of genetic manipulation is now less stringent than in the United States.

On large-scale production plants, for example, GMAG has taken the view that it is not feasible to enclose 100,000-litre fermentation vats in completely sealed containers or to operate them under negative pressure. Safety assessment, therefore, turns on an on-the-spot appraisal of the manufacturing plant and proposals for its

operation.

Although it is too soon to know whether GMAG will or should go out of business, the recruitment of specialists in genetic manipulation to the committee may now become increasingly difficult. The group, in any case, will have its hands full with the scrutiny of proposals for work in categories II-IV of the guidelines procedures, and with the principles for regulating large-scale manufacture. The reconstitution of the Dangerous Pathogens Advisory Group, expected soon, should, however, help to clarify GMAG's long-term future.

Engineering research

Planning for more

Washington

Federal policy-makers are now in the process of deciding whether the National Science Foundation (NSF) can provide an adequate framework for regenerating the health of the nation's engineering efforts. NSF officials are confident that it can. A prominent component of plans for reorganizing the foundation, formally presented last Thursday to the National Science Board responsible for foundation policy, is the creation of a new engineering directorate (see *Nature* 11 September). Applied science, which shares a directorate with engineering, would be distributed among the other research directorates.

Concern for the health of US engineering research has arisen as part of the wider debate about declining productivity and stagnating innovation. Equally important, however, has been what NSF acting director Dr Donald Langenberg describes as an "upsurge of interest" from engineering societies over the state of their profession and the role of federal support.

Both NSF and its critics agree that US engineering is in poor health. A rapidly escalating demand for engineers for large-scale construction projects in the oil and chemical industries has created a national shortage of engineers. This situation will be exacerbated by the demands of new plants to be financed out of the government's \$20,000 synthetic fuels programme.

The shortage is reflected in the high salaries paid to engineering graduates, while another result is that few graduates are tempted to stay on at university to do research once they have completed their first degree. Many postgraduate courses are now largely filled with foreign students and NSF estimates that, overall, there are 2,000 empty positions in university engineering faculties.

Some universities are designing schemes to compensate. Carnegie-Mellon University in Pittsburgh, for example, plans to offer loans of \$1,000 a month to PhD candidates, which need not be paid back if the candidate stays on and teaches.

However, there is general agreement that more than a piecemeal approach is needed;

the question is the form it should take. Dr Leo Young, president of the Institute of Electrical and Electronic Engineers (IEEE), has proposed a "blue-ribbon commission" — somewhat similar to Britain's Finiston Committee — to look at the problems facing the profession.

In Congress, discussion has focused on a bill introduced by Mr George Brown, chairman of the House science and technology subcommittee, which proposes setting up a National Technology Foundation. Mr Brown admits that his proposal has been put forward chiefly as a vehicle for discussing a range of proposals and that a revised bill is likely to be presented to the next session of Congress.

However, many engineers have not lost the opportunity to vent their frustrations at what they consider to be a lack of support from the federal government in general — and the NSF in particular — especially in fields outside space and defence research.

"Those university departments supported by the Department of Defense and the National Aeronautics and Space Administration have fared well, while civilian engineering, such as building technology and machinery design, have been neglected" complained Dr Bruno O. Weinschel, head of a private engineering company and secretary of IEEE.

National Science Board chairman Dr Lewis Branscomb of IBM admitted at congressional hearings on the bill that NSF support for engineering may have been weak in the past, but he insisted that this was now being remedied. He pointed out that for the past two years the board has proposed greater increases in the foundation's support for engineering research than for any other area of science.

NSF is hoping that its planned reorganization will go some way towards meeting its critics and will in particular head off any attempt to set up a new, separate institution (which might, in addition to the organization split, also be in a position to compete for funds). At a public meeting two weeks ago, held to discuss the reorganization proposals, Dr Langenberg said that engineering research could benefit financially from having its own directorate. He also hinted that reorganization plans were likely to be accompanied by a request for a significant increase in funds for engineering research when the foundation's budget request for 1982 goes to Congress in January.

Further financial commitments are likely to result from President Carter's decision about how to embrace science and engineering education in US schools and colleges, a report on which was sent to the White House by NSF and the Department of Education last month, and is expected to be made public shortly. But even if the Administration agrees that there is room for a larger role for engineering in the foundation, Congress may take some convincing. In debating the NSF appropriations in July, the House of Repre-

sentatives rejected a proposal for a 10 per cent increase in funds for the engineering directorate.

The White House report is likely to have a good deal to say about engineering research in American universities and colleges. Although, elsewhere, engineering graduates tend to stay away from doctoral courses, in its United States the demand is still quite high. There is, however, increasing concern about the willingness of potential faculty members to teach.

Perhaps more significantly, the powerful House Appropriations Committee claimed that its restriction of the use of NSF funds planned for various innovation projects was imposed "to ensure that an unacceptable level of unbudgeted items does not erode funding for basic research programmes which is — and continues to be — the *raison d'être* of the foundation".

These sentiments find an echo, though somewhat muted, within the scientific community. At the public meeting on 13 September, representatives of several of the NSF's advisory committees expressed concern that too great an emphasis on engineering and applied science could put a further squeeze on basic research. Dr Langenberg replied that he felt basic science was sufficiently robust not to be significantly threatened, but not everyone present was convinced. **David Dickson**

Electronic publishing

Keyboard papers

Those who wish to publish a research article in a journal, who are asked to referee a paper or who simply want to read what their colleagues have been publishing may in future have to turn to a computer terminal. The fully electronic scientific journal is still a few years off, however. But a group of British researchers is already beginning to consider its possibilities, in a project supported to the tune of £256,000 by the British Library.

The aim of the project, which begins in earnest in November, is to investigate whether electronic journals might ever be feasible, how much they would cost the user and the type of problems they would pose to users. The leg work is being done by groups at the University of Birmingham Loughborough University. The Birmingham group, under Professor P. Jarratt, will be providing the central computer facility and hardware and software to other participants in the project at a cost of £122,000. The Loughborough group, under Professor B. Shackel, will coordinate the setting up of an experimental electronic journal using the rest of the funds.

About 35–40 people dotted throughout universities in Britain will be collaborating. Each centre will provide its own terminal linked in to the Birmingham computer. The British Library funds will go towards

paying for time spent on the computer and telephone connection costs. Appropriately, the collaborators in the experiment have been drawn from the British community of researchers into computer human factors, which will form the subject of the journal.

The project is expected to last two years during which time each collaborator will be required to submit two papers to the journal for publication. Papers can be submitted directly via the computer system, can be sent to the editor, Professor Shackel, in a reasonably neat form for input into the system via a word processor or can be sent in perfect form to Birmingham for input through optical character recognition. Referees, who will be drawn from the same group of collaborators, will be informed that a paper is waiting to be read by messages which will appear on their Visual Display Units (VDUs) the next time they access the system. The submission and refereeing of a manuscript will be confidential to the editor and author or referee. Only when the manuscript has been accepted for publication will it be available to all collaborators in the system.

The British experiment is not the first of its kind. An earlier, similar exercise supported by the National Science Foundation in the United States ended in failure. The main problem was that people found the computer system difficult and time-consuming to deal with. At the end of that project, no paper had been submitted to the experimental journal, let alone published.

Professor Shackel is confident that the British experiment will not fall into the traps of the American one. His project, he says, has been designed to allow users a fair degree of flexibility, something which the Americans omitted. Although the ideal operating mode would be for everything to be done directly onto the computer, including the original writing of manuscripts, refereeing and editing, Professor Shackel acknowledges that this would be impractical for most people. His system allows authors to submit papers in several different ways, including the conventional way of sending a typed manuscript to him. Referees will also have the option of reading manuscripts directly on their VDUs or requesting that they be printed out.

Another aspect of the project's flexibility, says Professor Shackel, is that it will also investigate the possibilities of publishing scientific newsletters, annotated abstracts and reports of workshop conferences electronically, not just research papers. He also hopes to study the potential for greater cooperation between authors, referees and editors by using computer communications, and for increasing informal communication between scientists working on similar problems in different places.

Judy Redfearn

Soviet swindles

Degrees by stealth

Soviet ministries are killing the goose that lays the golden egg, as far as applied science is concerned. So says the prestigious weekly *Literaturnaya Gazeta* in the latest round of its press debate on the relative merits of pure and applied research.

Such debates are a common Soviet method of airing and channelling public opinion on problems of general concern ahead of decisions by the Party. The present debate was launched by *Literaturnaya Gazeta* in January of this year, as a Soviet version of C.P. Snow's "two cultures". In particular, it aims to investigate why public opinion considers applied research so much less prestigious than pure research. This is more than an academic question because, according to Party directives, all Soviet research should be aimed towards benefiting the national economy. However, at the Twenty-Fifth Party Congress (1976), Mr Brezhnev said that in the long run "there is nothing more practical than a good theory". And although the Soviet higher education system stipulates that new graduates must work three years in whatever job they are assigned, it is the most brilliant graduates (and those with special Party backing), who end up in the academic research posts.

To a certain extent, the division between pure research (carried out in the Institutes of the Academy of Sciences) and applied research (carried out in the "Branch Institutes" belonging to the various ministries) is a formal one. As I. Novikov, a Corresponding-Member of the Soviet Academy of Sciences, pointed out in the latest round of the debate, leading establishments such as the Kurchatov Atomic Energy Institute or the Central Institute of Aero-Hydrodynamics (the "cradle of Soviet aeronautics") fall outside the Academy structure. For the most part, however, conditions in the branch institutes bear no comparison with those of the Academy.

The working environment of the branch institutes was criticized by S. Kara-Murza, apparently representing the younger generation of scientists. He complained that it was difficult to maintain pride in one's work, and that team spirit was hampered by the custom of hanging up on the notice board graded assessments of researchers' creative potential. The ministries running the branch institutes seem to be trying to extend the principles learned in maximizing productivity in factories to the field of scientific research. The branch institutes also tend to be inward-looking, concentrating on intra-departmental communication and missing outside developments. One example of this is the institute that was supposed to be playing a leading role in stock-breeding and was found to be using methods of

biochemical analysis which dated from the 1920s and 1930s. The institutes run by the All-Union Academy of Sciences and the academies of the Union republics are less insular and benefit from communication with the world scientific community.

Meanwhile, it seems that some employees of the non-Academy institutes have found unauthorized outlets for their frustrated talents. These range from a racket in fake degrees and diplomas uncovered last year in Georgia and Azerbaijan to last month's revelation that the rector of a technological institute of fishery in Astrakhan had been diverting student housing funds to build luxury flats. Perhaps the most ingenious example of corruption so far comes from the Novgorod Polytechnic Institute and involved senior staff and some two hundred students. The staff applied for funds for research for which they had no facilities — the students were entered as "researchers", and received a sizeable share of the profits.

Vera Rich

Space shuttle

Delay costs money

Washington

Officials of the National Aeronautics and Space Administration (NASA) have expressed confidence that despite a tight schedule it should be possible to meet the planned date of next March for the first orbital flight of the re-usable space shuttle.

The delays in the schedule of operational flights have already caused problems for some of the shuttle's early commercial users faced with the alternatives of setting back their launch programmes or opting for more expensive expendable launchers.

Satellite Business Systems, for example, which plans to launch a series of satellites for information relay by private corporations, has recently decided to use a Delta rocket for the launch of its second satellite in 1981, rather than waiting for its first scheduled shuttle launch, originally booked for next March but since pushed back to 1983.

Even greater problems have been caused for Intelsat, the consortium of telecommunications authorities which pay for and use the communications satellites which are the present base of international telecommunications traffic.

Originally it had been intended to launch several of the Intelsat V series of satellites, the first due in December, from the shuttle. Now at least the first five out of eight launches will be from Atlas Centaur rockets.

Intelsat is keeping its options open. In addition to its shuttle bookings, and a recent decision to order the extra Centaur launches, space has been booked on two early Ariane launches (which remain dependent on the success of further tests of the European rocket).

For Intelsat V, the main problem caused by schedule uncertainties has been cost. Expendable launches are on average about twice as expensive as shuttle launches. For Delta payloads, for example, the relative costs would be \$21 million for a rocket launch compared with \$8 million to \$12 million for a shuttle launch, depending on the configuration in the shuttle bay.

But for the next generation of communications satellites, Intelsat VI, there is also a design problem, because payloads designed to fit neatly into the bay of the space shuttle, taking up the minimum length on which the cost is determined, may have a diameter too large to fit alternative launchers.

Already this has raised serious difficulties for the Hughes Aircraft Company, whose LEASAT communications satellite could face an expensive redesign if the shuttle launch schedule is not maintained. For Intelsat VI, it could mean delays in deciding the final design parameters. Intelsat VI satellites are to be bigger and better than the Intelsat V series, which are the first to be stabilized on three axes, and thus to be capable of directing radio beams at small areas — such as cities — on the surface of the Earth.

The outline design of Intelsat VI would provide the equivalent of 40,000 telephone channels (compared with 12,000 for Intelsat V). To economize in frequency spectrum (in the 6–4 GHz and 14–11 GHz bands) both series of satellites use circular polarization in opposite directions.

Operational specifications developed by Intelsat engineers were presented to the organization's board of governors in Washington this week. Given the uncertainties over the shuttle programme the board may decide to postpone approval of the specifications, perhaps until it meets again in December. Meanwhile the constraints on size and weight for Intelsat VI are being set by the capabilities of Ariane.

The delays in the shuttle programme are particularly embarrassing to NASA at a time when there is growing evidence that the demand for launches of telecommunications satellites will outstrip the supply of launchers well into the decade, even with both shuttle and Ariane operational and the maintenance of a fleet of expendable launchers.

Reflecting this demand, for example, NASA announced last week that eighteen space missions using expendable launch vehicles will be conducted by the end of September 1981, two carrying NASA scientific instruments and the other sixteen payloads for other government agencies and private companies.

Faced with the prospect of a full order book, NASA officials are keeping up the pressure for as early a shuttle launch date as possible. Despite renewed problems both with the engines and with the thermal tiles experienced over the summer, the agency has agreed to absorb the extra two months delay by squeezing the last planned six

months of work into four months.

So far, no further problems have been experienced, and Dr John Yardley, NASA Associate Director for Space Transportation Systems, said last week that progress on construction and testing was satisfactory and NASA is still aiming for a March launch date.

But there is still a long way to go before the shuttle can be launched. The final six week period, when many of the components will be tested together as a single system for the first time, will be crucial. So despite official optimism, no-one will be surprised if the launch slips back later into the summer.

David Dickson

Genetic manipulation

Ethics of change

Washington

Full-scale medical application of recombinant DNA technology to human genetic therapies may still be a few years off, but a Presidential Commission decided last week that it is close enough for federal agencies to prepare for the ethical decisions that research workers and medical practitioners may soon have to face. How, for example, should one balance the beneficial aspects of an attempt to correct a deficient gene against potential side effects of the treatment, or against the possibility of undermining traditional, often religious, conceptions of an individual's relationship to past and future generations?

Such broad philosophical questions have been discussed ever since manipulating the genetic make-up of human cells was first suggested. Temporarily eclipsed by the public debate over the immediate health hazards of recombinant DNA research, the wider questions have now resurfaced in the wake of the US Supreme Court's decision to allow the commercial patenting of microorganisms. Evidence for and against further federal involvement was heard last week by the President's Commission for the Study of Ethical Problems in Medicine and Biomedical and Behavioral Research, a body formed this year under congressional mandate.

The members of the commission felt that they lacked the time and expertise to study the whole range of ethical issues raised by genetic engineering. But they did agree to a survey by the commission's staff of the more immediate questions raised by the imminent application of recombinant DNA techniques to the treatment of a range of genetic disorders.

Even those members who were reluctant to accept the existence of major ethical problems accepted the case for educating the public to distinguish between topics that are of legitimate concern (such as potential medical side effects or deliberate misuse) and those that are not (such as the

creation of a race of monsters).

The principal stimulus for the commission's interest was a letter written to President Carter in July, after the Supreme Court decision, by the leaders of three prominent religious groups, the National Council of Churches, the Synagogue Council of America and the United States Catholic Conference. They expressed concern over possible dangers triggered by the rapid growth of genetic engineering and raised moral, ethical and religious questions over experimentation with humans. The commission thereupon asked the President whether it should study the ethical problems raised by genetic engineering, and earlier this month, Dr Frank Press, director of the Office of Science and Technology Policy (OSTP), suggested a survey of the current practice of federal agencies.

In a more expansive reply to Dr Claire Randall, general secretary of the National Council of Churches, Dr Press said he agreed that the Supreme Court decisions had significant implications and added that, with the legal barrier to patentability removed, there might be danger of proceeding too rapidly in the development of genetic engineering techniques.

Dr Gilbert Omenn, previously associate director of OSTP and now deputy director of the Office of Management and Budget, pointed out that many studies of the ethical issues were under way and, compared with more pressing problems of medical care, genetic engineering should be relatively low on the commission's list of priorities.

Commission member Dr Arno Motulsky suggested that the application of recombinant DNA techniques in, for example, the treatment of sickle-cell anaemia was one of a whole series of advances in medicine which "do not raise particular complications". However, Dr Richard Roblin of the Frederick Cancer Research Center predicted that "within three to five years a hospital committee will have to decide whether to give the go-ahead for the functional use of gene therapy". Such gene transplants raised the need for criteria to distinguish legitimate from illegitimate forms of controlling genetic material, particularly where the latter might be seen as a new form of eugenics.

The commission itself was split over the seriousness of the ethical issues, but it agreed on three points: that the public needed guidance in distinguishing reasonable from unreasonable concerns; that enough scientific and medical data now exist on which to base a substantive discussion of the short and medium-term applications of genetic engineering in medicine; and that, apart from studies by the Office of Technology Assessment and the National Science Foundation, other federal agencies had focused primarily on the safety or patent aspects of recombinant DNA research, addressing ethical issues only subordinately.

The commission's staff study is likely to

suggest how agencies could do more. And this would require a response, in particular from the National Institutes of Health (NIH), which have broad responsibility for stimulating the applications of research results through technology transfer.

It will not be easy to develop guidelines on ethical issues comparable to those which exist on safety. The federal government has no jurisdiction over the treatment that a private medical practitioner can offer, while the separation of church and state is paramount.

David Dickson

French Science

Same now, more later

Paris

The French budget for science will grow with inflation next year — but it is not quite the bonanza for science that the Secretary of State for Research, M. Pierre Aigrain, was claiming last week.

At a press conference to launch the proposed budget, which goes before parliament at the end of next month, M. Aigrain boasted of an increase of 20.4 per cent in non-salary research spending in 1981. Inflation is running at 14-15 per cent, however, and some of the increase will be delayed until 1982.

Nevertheless, M. Aigrain's news is good news. In the past two years, the minister has lost most applied research, accounting for about half his budget, to the control of the minister of industry, M. Giraud, and there were fears that one result would be reduced growth in the budget for basic science. But in the event, Aigrain said last week, basic science will grow by four per cent more than applied.

The 1981 budget is highly selective, and some areas of research will shrink in real terms. Space research funded by the Centre National d'Etudes Spatiales, for example, will suffer an increase of only 9 per cent in money terms, while FF535.6 million (£54 million) available to Aigrain's own office, the Délégation Générale à la Recherche Scientifique et Technique, for making direct research contracts with universities in 1980 will actually fall to FF471.7 million in 1981. (It should rise again in 1982.)

Some sleight of hand with the budget conceals these facts. Running costs plus "programme authorizations" will indeed grow by 20.4 per cent next year but authorizations include commitments for future years. Actual cash for basic research is planned to increase from FF3,630 million in 1980 to FF4,200 million in 1981 — a growth of only 15.7 per cent, and of the same order as inflation. Nevertheless, the budget commits the government to more spending in 1982. Some important institutions will even do well in 1981.

The main beneficiaries affecting universities are the three principal research agencies, the CNRS (Centre National de la Recherche Scientifique), INRA (Institut

National de la Recherche Agronomique) and CNEXO (Centre National pour l'Exploration des Océans); the fourth, INSERM (Institut National de la Santé et de la Recherche Médicale) just keeps pace with inflation. One objective is to help to renew ageing equipment and INSERM is judged to have done well in this regard in past budgets. CNRS, which with some 8,700 scientists runs many of the leading laboratories, gains 24 per cent in its "operational" budget, INRA 31 per cent and CNEXO 34 per cent.

Certain big projects also do well. There will be FF48.5 million to cooperate with the Soviet Union on a Venera probe to Venus and a FF64.3 million contribution to the European Space Agency Spacelab programme. The budget also allows for the

Soviet bomb scare

Washington

Reports of a possible violation by the Soviet Union of a 1974 agreement on underground nuclear tests could prejudice negotiations on a Comprehensive Test-Ban Treaty, now expected to be concluded within a few months.

Officials in the Carter Administration are hurriedly trying to check preliminary seismological data indicating that the 1974 agreement, under which the United States, the Soviet Union and the United Kingdom agreed not to conduct underground tests equivalent to more than 150 kilotons of TNT, may have been violated.

According to a report in the *New York Times*, unnamed security aides claim that an explosion detected on Sunday, 14 September, was the largest that had been registered since the agreement came into effect in 1976. Some said it was close to, but still above, the maximum permissible limit; others that it may have been as large as 650 kilotons.

Data are now being collected from as many detection stations as possible to see if this assessment is correct, or if stories in the press merely reflect attempts to discredit the Administration's arms limitations efforts.

Officials in the Office of Science and Technology Policy said last week that it would be about a month before sufficient data were collected to make a reasonable judgement on the size of the explosion, but the Administration's concern has already been expressed by the State Department to the Soviet ambassador.

Current negotiations aimed at securing a comprehensive test-ban have become bogged down over defining the limits beneath which it may be impossible to distinguish a man-made explosion from normal seismic activity. In recent weeks, negotiators in Geneva have been expressing optimism that agreement was close, and that a draft treaty could be ready for signature within a few months.

David Dickson

construction of a submarine and other work (FF21.7 million); a start on construction of a fusion reactor (TORE SUPRA) at Cadarache (FF15.5 million) and a start on a hot dry rock geothermal project (FF1 million). In basic physics, the government will contribute to the "second souffle" at the Institut von Laue Langevin (FF5.4 million), the heavy ion accelerator GANIL (FF6.7 million) and the Franco-German Institut de Radio-astronomie Millimetrique (FF22.6 million).

One tangible benefit of the budget will be the creation of 625 new junior posts, 410 of them for scientists and the rest for technicians, engineers and administrators.

The budget also reveals the French commitment to defence research, spending on which has grown by 23 per cent a year since 1977 to reach a level of FF11,000 million in 1980, accounting for 37 per cent of the national spending for research and development. Of this fraction, some 28 per cent is described as "research" proper but only one per cent of this finds its way to universities.

Robert Walgate

German science

Frontier talk

An agreement on scientific cooperation between the two Germanies might well be feasible, according to Professor Kurt Hager, Chairman of the Education Committee of the Politburo of the German Democratic Republic (GDR). He was addressing the country's Fifth Conference on Higher Education (4-5 September) which was convened to discuss the contribution of universities and other higher educational institutions to strengthening the GDR's economic performance in the 1980s.

The suggestion carried the political proviso that development of relations "would be made easier if the responsible circles in the Federal Republic could bring themselves to abandon their . . . non-recognition of GDR citizenship and disregard for the sovereignty of our state".

The conference was attended by some 4,000 delegates from both academic staff and students, and it was not entirely a political exercise. Educational planners in the GDR have recently been paying considerable attention to the training of young scientists. Earlier this year, a working group from the Education Committee of the Politburo visited a number of educational establishments throughout the country. Following the presentation of their findings, Professor Hager had stressed that the early training of young scientists was a "vital task of far-reaching importance which would have considerable influence on further economic growth". He advocated that steps should be taken to ensure that "specially talented young people fitted for science" are discovered as early as possible.

Vera Rich

CORRESPONDENCE

Geothermal power

SIR—The recent News and Views article by Richardson and White (*Nature* 10 July)¹ and correspondence in your columns² have been concerned with the relative merits of two ways of using geothermal energy — district heating and electricity generation — and there does not seem to be much to choose between them. According to Richardson and White, “Neither of the schemes is particularly attractive in absolute terms”. Certainly, electricity generation directly from a geothermal source at a temperature below 200°C has previously been considered to be uneconomic^{3,4}.

It was because of this view that Patscentre, some three years ago, proposed a scheme that allowed the possible exploitation of such low-temperature geothermal sources for economical electricity generation if they coincided with sites for conventional power stations, existing or prospective⁵. Geothermal hot water could be efficiently used for feedwater heating in the power stations. I suggested that such a coincidence of sites might be found on the Hampshire (geological) sedimentary basin. The idea was taken up by the Central Electricity Generating Board, and a borehole was sunk last year at Marchwood power station, near Southampton. Earlier this year, you reported that an aquifer had been struck (*Nature*, 8 May)⁶. I understand that the geothermal hot water will probably be used in the Marchwood station as an experimental illustration of the proposed scheme.

It is to be hoped that, if successful, this experiment will be the forerunner of an acceptably efficient method of geothermal electricity generation in the UK and elsewhere.

R.V. HARROWELL

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SIR — The method of evaluation for the district heating option used by Richardson and White in their article on the use of UK geothermal energy resources (*Nature* 10 July, p.103) is not appropriate for UK geothermal resource conditions.

The Department of Energy Geothermal Energy Group have predicted that geothermal energy sedimentary basin resources in the United Kingdom will be most commonly in the range 65 to 80°C. We have recently completed a study for the Department of Energy examining the use of UK geothermal energy resources which concluded that a supply temperature of 65°C will be adequate to meet space and water heating needs. Underfloor heating systems or large natural convectors could operate with a supply temperature of 65°C to satisfy comfort conditions and achieve a return temperature of 30–35°C. We believe, however, that the optimum solution will arise from forced air convection equipment which could reduce the return water temperature to the geothermal source heat exchanger to 20–25°C and furthermore a proposal is in hand directed by W. S. Atkins and Partners and supported by a major UK heating equipment manufacturer to develop a range of such equipment at competitive price levels

when compared to standard temperature equipment.

A further important factor to be considered is that the depth of aquifers containing brine at 70°C is such that it could significantly reduce the cost of the geothermal well compared to an aquifer supplying water at 100°C. We suggest therefore that the findings of Richardson and White be reconsidered.

E. J. ATHONY

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Models of psychosis

SIR—In a recent News and Views article (*Nature*, 5 June)¹, Snyder expressed the view that phencyclidine — currently all the rage among drug-abusers in the United States — might provide a good pharmacological model of schizophrenia. In the course of his paper he makes a comparison with LSD-25 which, he suggests, is not a good model because it produces perceptual disturbances that are mainly visual in nature, whereas those found in schizophrenia occur primarily in the auditory modality. There is actually no factual basis for that statement; on the contrary, several studies (for example, Young²) indicate that the effects of LSD on perceptual, as well as on other psychological, functions, are remarkably similar to the symptoms of schizophrenia. This widely quoted misconception about LSD is only one of many which have led, in my view, to the drug being prematurely rejected as a possible pharmacological model for human psychosis. As I have argued in detail elsewhere³, I believe a good case can still be made for LSD; though Snyder may be right — phencyclidine may be better.

G.S. CLARIDGE

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University of Oxford, UK

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Educative computers

SIR — We were very interested to read your article “Money to spend” (*Nature* 21 August, p.750) about the government’s programme on microelectronics in schools and colleges.

One matter of fact, however, we feel should be put right. The estimate of about one hundred schools owning their own computers is a long way off the mark. A survey carried out in the first three months of the year by ourselves and the Schools Council, in which replies were received from 60 out of 104 local education authorities in England and Wales, showed 663 secondary schools with at least one microcomputer. (In Scotland, the figure at the end of August was over 250.)

Even those figures should be taken as very conservative estimates since numbers are growing rapidly from day to day. We know of one local authority, for instance, which is installing a new machine at its schools at the rate of one a week.

If any of your readers would be interested to have a copy of a brief report of our survey referred to above, we should be pleased to send them one.

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Interferonology

SIR — I am writing in response to the note on interferon nomenclature which appeared recently in *Nature* (10 July 1980; **286**, 110).

I recognize that the previous nomenclature is less than ideal because the names (“leukocyte”, “fibroblast” etc.) now refer to molecular classes of interferons rather than tissues of origin.

With the foregoing qualification in mind, what is gained by replacing L, F and T with α , β and γ ? If physicists changed the names of the u , d , s and c quarks, let us say, to x , y , z and t , absolutely nothing would be gained except the additional complication of memorizing which is which. Fortunately, most physicists, unlike most interferonologists, are aware that such symbols are merely arbitrary denotations for distinct entities and are in themselves meaningless. (As long as there are three interferons, why not Athos, Pothos and Aramis? D’Artagnon would be available if another were discovered.)

Until the structures of interferons are better understood individually and comparatively so that an informative nomenclature can be devised, I fail to see the wisdom in replacing Roman letters with Greek, in substituting one purely arbitrary system with another equally so, and in abandoning a nomenclature which, at least, has the advantage of familiarity.

LEE H. KRONENBERG

San Diego, California

Educating scientists

SIR — I fear that your readers may mistake your report strangely entitled “Subject in search of discipline?” (*Nature* 31 July, p.432) for a factual account of Sir Alec Cairncross’s report to the Nuffield Foundation on “Science Studies”. *Nature* suggests, not least by the tone of the article, that Cairncross’s report is critical of “Science Studies”. May I remind you of a major recommendation of the report? Cairncross declares that “There is a continuing need for injecting into most science degrees some provision for minor courses covering up to 15 per cent of the curriculum. These should be examinable”.

This is strangely at odds with what *Nature* has to say. *Nature* associates the minor courses to which Cairncross refers with “general chat about the social relations of science”, suggests that “Much of what is now provided for students under this heading centres around the much talked of Promethean dilemma” and that “teachers are all too often bent on grinding other axes — political, ideological or religious”. These assertions are not drawn from the Cairncross report and neither are they true. They are uncomfortably close to the sorts of uninformed prejudices which many of us involved in serious attempts to serve science education have long struggled to overcome.

Nature expresses itself in favour of the enrichment of science education. I am profoundly sorry that it has not chosen to treat more seriously those who believe that they are making some progress towards this end.

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NEWS AND VIEWS

The last neanderthal in France?

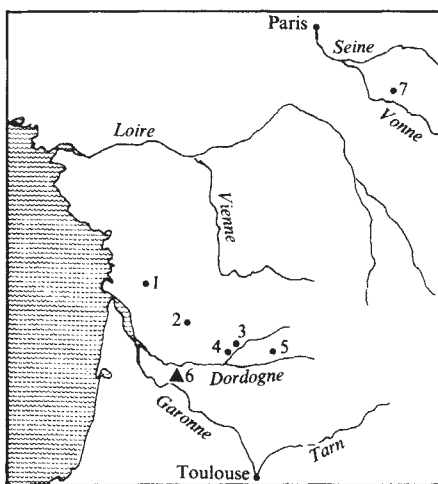
from A.M. ApSimon

THE discovery by Lévêque and Vandermeersch¹ of the fragmentary remains of a skeleton of classic neanderthal type in a Châtelperronian archaeological layer at Saint-Césaire (Charente-Maritime, France), raises once again the problems of the replacement c. 34,000 BP of the neanderthal population (*Homo sapiens neanderthalensis*) of western and central Europe by a population of sapiens men (*Homo sapiens sapiens*). Three explanatory hypotheses have been proposed: (1) straightforward evolutionary transformation *in situ* of neanderthal into sapiens; (2) immigration of sapiens leading to the extinction of the neanderthals; (3) sapiens immigration followed by interbreeding in which the distinctive characters of the neanderthals were lost.

Before this new discovery, neanderthal finds from France were exclusively associated with the earlier, Mousterian stone industries (Middle Palaeolithic) characterized by a rich variety of scrapers, knives, spear points and other tools, made mostly on flakes and probably struck using stone hammers. In contrast sapiens were associated with lithic industries (Upper Palaeolithic) in which distinctive long narrow flakes (blades) probably struck off using antler punches played an important role. Although the difference in the industries associated with sapiens and neanderthal suggest they evolved separately, the possibility of *in situ* transformation is not excluded because there are long periods in which the stone industries are without distinctive human remains. In particular, one of the many variants of the Mousterian industries, the Mousterian of Acheulian tradition (MTA) is notorious for its lack of any morphologically distinctive human remains.

Study of the stratigraphic relationships of the different variants of Mousterian industry in SW France² suggested that the MTA was relatively late, whereas the known neanderthal remains were relatively early within the earlier part, Würm I-II, of the last glaciation. This would have left a period up to 20,000 years long (c. 55-34,000 BP) from which no human remains were known and during which the transformation to sapiens morphology could have taken place.

Refutation of this argument has come from H. Laville's elucidation³ of the relative chronology of the sedimentary and climatic phases, as well as of the associated Mousterian industries and human remains, of the early Würmian (Würm I and II) deposits of the caves and rock shelters of the Perigord region of SW France. This has shown that a number of well-known neanderthal finds, including Le Moustier, La Chapelle-aux-Saintes, La Ferrassie and La Quina, date from the later sub-stage



Map showing location of finds of human fossils in France referred to in text.

1. St. Césaire 2. La Quina 3. Le Moustier 4. La Ferrassie 5. La Chapelle-aux-Saints 6. Combe Capelle 7. Arcy-sur-Cur neanderthal • sapiens ▲.

(Würm II). Guichard⁴ has used this chronology to reassert the coexistence of the four main Mousterian variants, including MTA, during most of the earlier Würmian. F. Bordes⁵ had suggested that these variants were the culturally determined markers of distinct ethnic groupings and that evolutionary divergence between these groups could have allowed the emergence of the sapiens lineage, perhaps among the makers of the MTA variant, since this variant showed features foreshadowing Upper Palaeolithic industries. Criticism of Bordes' hypothesis has centred on the extreme improbability of the conservation of separate lineages in

an area as small as Perigord through the 40,000 year long early Würmian stage.

L. and S. Binford⁶ attempted to break free from the impasse of ethnic *versus* chronological explanation for variability in Mousterian industries, by suggesting that it might be better explained by adaptation to changing environments and by different tool kits being used for seasonally or functionally different activities. Although the Binfords' hypotheses have been faulted by Bordes⁷ and others⁸ on grounds that include the unrepresentativeness of the material analysed by them, the over-reductive nature of their analysis, the appearance of similar industries in dissimilar environments, the occurrence of long-maintained year-round occupation and the absence of difference between tool kits from open air and from rock-shelter sites, their work remains of capital importance in calling attention to the potential complexity of causal explanations for artefact variability.

Guichard⁴ points a way out of this dilemma through his re-analysis of the diagnostic criteria for the different Mousterian industrial facies. This shows that elements are very rarely entirely absent from a given facies, even though the frequency of particular elements may be very low. This has two consequences: first, it serves to reaffirm the essential unity of the Mousterian tradition and warns against reification of the differences discerned by typologists and, second, it removes the necessary implications of ethnic or functional interpretation, and further weakens the argument for *in situ* transformation.

C.B. Stringer's multivariate metrical study of later Pleistocene hominid cranial material⁹ also speaks against the idea of transformation since his results show that the classic neanderthals, including those dated to 'Würm II' in France, were less like the sapiens population of Upper Palaeolithic Europe than were the early neanderthals. His conclusion is that they were probably not the ancestors of modern man.

Although the Châtelperronian is usually treated as the first stage of the Upper Palaeolithic in France, Bordes showed that it probably developed from late Mousterian industries, a possibility hinted

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Climatic Phases	Age BP	Human fossil finds	Industries
Würm III	23,000	Combe Capelle?	Peregordian & Aurignacian
Würm II — III	29,000	St. Césaire? Arcy?	Aurignacian & Châtelperronian & Mousterian
	34,000		
Interstadial	39,000		
Würm II	55,000	La Ferrassie, La Quina La Chapelle-aux-Saints (?) Le Moustier (?)	Mousterian
Würm I — II Interstadial			

Chronological table showing earlier last glaciation phases in SW France (simplified, after Laville³ and others.

Transition between Middle and Upper Palaeolithic industries

(Châtelperronian is considered the initial stage of Upper Palaeolithic because of blade technology, Aurignacian is marked by bone spear points and specialised flint scrapers and graters, Perigordian by developed blade technology. Szeletian (see text) uses leaf shaped flint spearheads and is technologically transitional from Middle to Upper Palaeolithic.)

at by cases of direct superposition of the two industries. Thus the Saint-Césaire discovery need come as no surprise in view of previous suggestions that isolated molar teeth possessing very large crowns with complicated relief found in the Châtelperronian deposits at Arcy-sur-Cure, Yonne (Grotte-du-Renne, layer XI), might be neanderthal rather than sapiens. The only other human fossil for which association with Châtelperronian has been claimed is the fully sapiens skeleton from Combe Capelle. However, an objection has been raised by Asmus¹⁰. Noting that the Châtelperronian layer in which the skeleton lay was very shallow, she suggests that the skeleton could only have been protected from disturbance if, when the burial was made, some at least of the later, overlying Aurignacian layer was already present (for further discussion see ref. 11).

Radiocarbon dates for Châtelperronian lie between c. 34,000 and 31,000 BP, so that the Saint-Césaire find is probably very close in time to the appearance of sapiens men. Sapiens remains occur in Aurignacian deposits for which radiocarbon dates of c. 32-31,000 BP are available. Rigaud¹² takes cases of interstratification as showing the contemporaneity and separate identities of Châtelperronian and Aurignacian in both their initial and developed stages. If the extant human remains are representative then there was a period during which neanderthal and sapiens men coexisted in SW France. If the rate of morphological change which this situation would require decisively rules out *in-situ* transformation, we are left with the choice between hypotheses two and three.

For indications as to where our choice should lie, we may usefully turn to evidence from recent hunter-gatherer groups. This suggests that both neanderthal¹³ and early

sapiens may have been organized in small bands, moving within limited but loosely defined territories and operating open, inter-band mating systems. Where bands were moving in areas not fully occupied by defined territories, contacts with previously unknown bands may have happened from time to time. Initial signalling between groups would probably be visual, and if contact behaviour proceeded along simple formalized lines, difficulties in verbal signalling need not have prevented peaceful contacts between strange bands. Châtelperronian — Aurignacian interstratification and possible artefact interchange suggests that peaceful contact may have been the rule, in which case there is no necessity to postulate inhibitions to neanderthal — sapiens mating. As the present chronology is only reliable to within about a 1,000 years the disappearance of the distinctive cranial features of the neanderthals would appear to be virtually instantaneous. The recovery rate of fossil human remains is so low that the chance of being able to document the change is very small.

To suggest that hypothesis three is the preferred one is not to solve all our problems, for despite new evidence of the evolution of *Homo sapiens sapiens* outside Europe¹⁴ there is no good evidence to show how they spread to central and western Europe by c. 34-31,000 BP, nor is there evidence of their appearance in east-central Europe earlier than in France. Those sapiens finds from central Europe which by their attribution to Würm II (for example Mladeč, Moravia) have been supposed to provide such evidence, simply reflect differing applications of the Würm I-II-III system in the two areas and it is clear that they belong to what would be called Würm III in SW France.

Nor is there evidence that the Aurignacian complex appears earlier either in central Europe or in the Near East than it does in France. Dates in the range 40,000-35,000 BP quoted for Upper Palaeolithic assemblages in the Mediterranean and Near Eastern region depend on extrapolation of observed sedimentation rates in cave deposits and cannot be taken as a reliable indication of the origins and previous development of 'the Upper Palaeolithic' in that region. Many workers have seen reminiscences of the Mousterian in the Aurignacian, even though in France there is no evidence of interstratification and only very doubtful evidence of end-to-end contact. In central Europe the Szeletian industry appears to evolve from Middle to Upper Palaeolithic status and association with both neanderthal and sapiens remains has been reported. It is uncertain whether the bone spearheads and Aurignacian elements in the evolved Szeletian reflect contact with contemporary Aurignacian bands, or whether, as Hahn¹⁵ suggests, the Aurignacian can be regarded as a logical continuation of the Mousterian and Szeletian tool kits. In his view some features of Aurignacian assemblages were already present at a low frequency in Mousterian assemblages, increasing in Szeletian assemblages, so that these show a crossover between decreasing frequencies of Middle Palaeolithic and increasing frequencies of Upper Palaeolithic elements, in particular the bone tools. The coincidence of the appearance of sapiens men with the Aurignacian in Europe is hard to resist, but it may still be a coincidence, and in the Near East, sapiens men already occur with Mousterian industries¹⁶ as at Skuhl and Qafzeh in Israel. We should be wise to maintain with Maheu¹⁷ "the denial of any causal connection between physical characteristics and industry or culture" and to refuse explanations for the disappearance of neanderthal man which rely on unverifiable assumptions of the inferiority of his intellectual and social capabilities. □

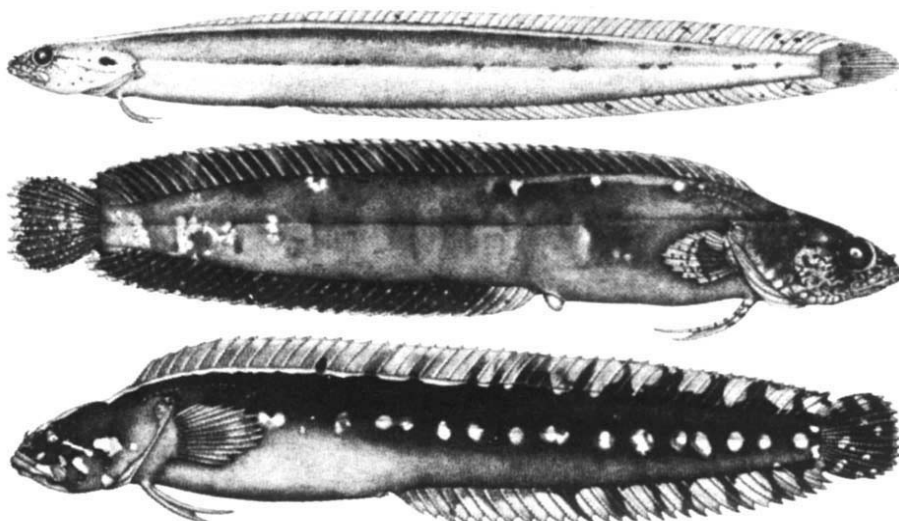
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Snakeblennies

from Alwyne Wheeler

The snakeblennies (fishes of the tribe Ophiclinini within the family Clinidae) are small (up to 16 cm long) and live in shallow water along the cool, southern coasts of Australia. They can be captured in tidal rock pools and in bays at depths of 13 m and several species are known to burrow in mud.

A recent taxonomic study by George and Springer* has made a considerable contribution to our knowledge of these fishes, not least because they name no fewer than five previously undescribed species. They have also revised all the previously named species and produced a clarification of the relationships of these little-known fishes. Thus, the species *Peronedys anguillaris* which was previously assigned to a family on its own, and the genus *Sticharium* which hitherto had been placed in the Notograptidae, are now combined with the Ophiclinidae. These fishes are not, however, considered to be distinct from the members of the family Clinidae, which are widely distributed through the tropical and subtropical oceans. For this reason, these southern Australian fishes



Ophiclinops hutchinsi, new species (89.0 mm); *Ophiclinus pectoralis*, new species (52.0 mm); *Ophiclinus gracilis* (68.0 mm). Drawings by J.R. Schroeder.

are placed in a tribe Ophiclinini within the Clinidae.

One of the pieces of evidence which substantiates the close relationship of *Peronedys* (which was thought to be distinguished by having no pectoral fins) is that several of the species of *Ophiclinops* have very minute pectoral

fins, including the newly described *Ophiclinops hutchinsi* from Western Australia.

*Anita George & Victor G. Springer: Revision of the clinid fish tribe Ophiclinini, including five new species, and definition of the Family Clinidae. *Smithsonian Contributions to Zoology* 307 (Smithsonian Institution Press, City of Washington, 1980).

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Sexual dimorphism and the evolution of higher primates

from R. D. Martin

In this issue of *Nature* Fleagle *et al.* report that three species of primate from Oligocene deposits at the Fayum fossil site show evidence of sexual dimorphism. This discovery has important implications for our understanding of the radiation of monkeys, apes and man as well as enabling us to guess something of the social structure of the earliest known Old World anthropoids.

The Fayum fossil site in the Egyptian desert south-west of Cairo is of special significance for the reconstruction of primate evolution. Vertebrate fossils from these Oligocene deposits, dating back some 30 million years, include the first known representatives of the 'higher' primates (monkeys, apes and man). In fact, in the entire African continent, fossil primates from this site provide the only undisputed palaeontological evidence of primate evolution for the first half of the Tertiary epoch (Palaeocene–Eocene–Oligocene). The first fragmentary jaws and teeth of Fayum primates were recorded about 75 years ago and a series of collecting expeditions led by Elwyn Simons (then of Yale University) in 1963–1967 produced a wealth of fossil material from five primate genera (*Apidium*; *Parapithecus*; *Oligopithecus*; *Propliopithecus*; *Aegyptopithecus*). But soon afterwards events in the Middle East led to suspension of all palaeontological work in the Fayum, and it

was not until 1977 that a new programme of joint expeditions was organised by the Geological Survey of Egypt and Duke University. The new initiative has yielded additional material throwing new light on the early evolution of the higher primates.

Interpretation of the initial radiation of the monkeys, apes and man has been thrown into turmoil by new information on continental drift. The traditional view, assuming stable continental positions is that the New World monkeys (platyrrhine) and the Old World monkeys and apes (catarrhines) are two quite distinct branches derived from a prosimian stock spanning the northern continents. The platyrrhines and catarrhines are held to have attained the 'simian grade' independently through parallel evolution. The acceptance of continental drift has, however, generated increasing support for the alternative view that the platyrrhines and catarrhines shared a common ancestor in Africa which had already attained the simian grade. Opponents of this interpretation point out that the first fossil evidence for New World monkeys appears only in the late Oligocene and that the South Atlantic undoubtedly represented a formidable barrier to invasion by terres-

trial mammals at that stage. Conversely, reconstructions of continental positions indicate that invasion of South America from North America would have been at least equally unlikely during the Oligocene.

In all of this, the Fayum primates occupy a central position as the earliest relevant fossils. Interestingly, they do show numerous cranial and postcranial similarities to modern New World monkeys, but this is compatible with either monkey or ape origins since the similarities concern primitive features only to be expected in early representatives of the simian grade. It is therefore crucial to determine whether the Fayum primates exhibit specializations specifically linking them to Old World monkeys and apes. On the basis of previous evidence, Simons and his colleagues has already suggested this to be the case. Indeed, Simons proposed that the fossils could be divided into two groups (*Apidium* and *Parapithecus* vs *Oligopithecus*, *Propliopithecus* and *Aegyptopithecus*) related to the origins of the Old World monkeys and apes, respectively.

The new primate fossil material from the Fayum deposits has produced no great surprises in taxonomic terms; all of the new specimens can be confidently allocated to four of the five existing genera (*Apidium*; *Parapithecus*; *Propliopithecus*; *Aegyptopithecus*). But the increased sample sizes

for the dentitions of three species (*Apidium phiomense*; *Propithecus chirobates*; *Aegyptopithecus zeuxis*) have permitted identification of a marked degree of sexual dimorphism (Fleagle, J. G., Kay, R. F. & Simons, E. L. this issue of *Nature* p328). For each of these species, the molar teeth of the lower jaws seem to fall into uniform series, whereas the lower canines and anterior premolars show a bimodal size distribution. (The anterior lower premolar has a facet for occlusion with the upper canine and therefore reflects the degree of development of that tooth.) Of course, one must be wary of inferring sexual dimorphism in fossil material, since two separate but closely related species may be involved, and past controversies over the relationships between gracile and robust australopithecines serve as an object lesson here. Fleagle *et al.*, to their credit, made a separate approach by considering the intraspecific coefficient of variation for the depth of the mandible, which was found to be correlated with the degree of body weight dimorphism in a sample of living primate species. On this criterion, *A. phiomense* and *A. zeuxis* were both found to be comparable to modern sexually dimorphic species such as the howler monkey, the proboscis monkey and the gorilla. It is therefore reasonable to conclude that there is good evidence for sexual dimorphism in at least three of the Fayum primate species.

The phenomenon of sexual dimorphism in body size and development of the anterior dentition in living primates has been examined in detail by several authors in recent years (Gautier-Hion, A. *Mammalia* 39, 365; 1975; Leutenegger, W. & Kelley, J. T. *Primates* 18, 177; 1977; Clutton-Brock, T. H. & Harvey, P. H. *J. Zool.* 182, 1977; Clutton-Brock T. H., Harvey, P. H. & Rudder, B. *Nature* 269, 797; 1977; Harvey, P. H., Kavanagh, M. & Clutton-Brock, T. H. *J. Zool.* 186, 475; 1978). The following important generaliza-

tions about sexual dimorphism have emerged from these quantitative studies: (1) It is always lacking in species living in monogamous family groups; it may or may not be present in species where males have breeding access to more than one female (polygyny); (2) It tends to increase with increasing body size; (3) It is especially prevalent among terrestrial primate species; (4) It is virtually absent among living prosimian primates, uncommon in New World monkeys and markedly present only among Old World monkeys and apes.

From these generalizations it follows that the three apparently sexual dimorphic Fayum primate species were closer to modern Old World monkeys and apes than to modern New World monkeys. In the New World, notable sexual dimorphism is present only in the large-bodied howler monkeys (*Alouatta* spp.), with males weighing approximately 30% more than females. Yet the Fayum primates span virtually the same body size range as the modern New World monkey Family Cebidae (600 g — 6 kg; Kay, R.F. & Simons, E.L. *Int. J. Primatol.* 1, 21; 1980) and exhibit signs of sexual dimorphism even at the lower end of that size range (*Apidium phiomense*). Kay and Simon (*loc. cit.*) have also inferred from the relatively tall molar teeth of one of the Fayum species *Parapithecus grangeri*, that it might have held terrestrial tendencies, though unfortunately there is insufficient dental material to show whether this species was sexually dimorphic. The prevalence of sexual dimorphism (even at quite small body sizes) and the widespread adoption of terrestrial tendencies among the Old World monkeys and apes are probably linked features, and the Fayum primates seem to fit the general pattern.

Further, it seems highly unlikely that the sexually dimorphic Fayum species lived in monogamous family groups and their social systems probably had a polygynous

base, as is typical of modern Old World monkeys. Among modern New World monkeys, by contrast, monogamy is common and it has been suggested that this could have been the ancestral condition for platyrrhines. Thus, the new evidence uniformly suggests that the Fayum primates were an integral part of the evolutionary radiation of the Old World Monkeys and apes, as has been consistently maintained by Simons. One must remember, though that the common ancestor of all monkeys, apes and man must accordingly have antedated the Fayum deposits. Palaeontologists generally tend to date the origin of any group from the first known fossils, whereas incompleteness of the fossil record (particularly for Africa during the first half of the Tertiary) allows for a much earlier date. The earlier the actual date of divergence of the platyrrhines and catarrhines, the greater is the likelihood of an African origin of the higher primates.

An understanding of the the relationship between primate sexual dimorphism and social organization is of considerable interest and enables us to suggest the kind of social groups that fossil primates may have lived in. Explanations of why sexual dimorphism evolved have, hitherto, invoked two male-oriented causative influences: (1) intense male-male competition for females, and (2) defence of the social group against predators by large males. However, sexual dimorphism involves divergent male and female strategies and little attention has been given to factors which might favour small body size in females. In an elegant analysis of dental sexual dimorphism in primates, Harvey *et al.* (1978, *loc. cit.*) used relationships between female tooth size and body size as baselines for assessing male dental development ('relative male tooth size') and were able to analyse more precisely the factors associated with exaggerated development



PHYSICS WITHOUT APPARATUS

Acoustical experiments require, for the most part, the aid of some good instrument or valuable piece of apparatus. Nevertheless a few instructive illustrations of the principles of the science can be improvised without difficulty. Firstly, there is the familiar experiment brought into fashion, we believe, by Prof. Tyndall, of setting a row of ivory billiard balls or glass solitaire marbles along a groove between two wooden boards, and showing how their elasticity enables them to transmit from one to another a wave of

moving energy imparted to the first of the row, thus affording a type of the transmission of sound-waves from particle to particle through elastic media. Then we may show how sounds travel through solid bodies by resting against a music-box or other musical instrument, a broomstick, or any convenient rod of wood, at the other end of which we place our ear. A kindred experiment, illustrative of the transmission of sounds through threads, is depicted in the Figure. A large spoon is tied to the middle of a thin silken or hempen thread, the ends of which are thrust into the ears upon the ends of the thumbs. If the spoon be dangled against the edge of the table it will resound, and the tones reach the ear like a loud church bell. The thread telephone or "lover's telegraph," is upon the same principle, the thread transmitting the whispered words to a distance, without that loss by spreading in all directions which takes place in the open air.

From *Nature* 22, 23 September, 488, 1880.



of the anterior teeth in males of certain species (e.g. terrestrial vs. arboreal species and polygynous vs. monogamous species).

Their data also show that, at any given body size, males often have a shorter row of cheek teeth than females, suggestive of lower energy requirements. Significantly, this applies only to species with polygynous social systems and the divergence between males and female increases with increasing body size. Harvey *et al.* briefly mention the possibility that sexual dimorphism may be related to differential dietary strategies of males and females, and the cheek tooth data indicate that this deserves close examination. It has already been shown, through indirect computations based on

activity budgets recorded in captivity, that female Sykes monkeys (*Cercopithecus albogularis*) may consume up to 30% more energy than a male, despite the fact that the latter is some 40% heavier (Coelho, A. M. *Primates* 15, 263, 1974). It is conceivable that divergent energy strategies of females and males may be the primary factor underlying sexual dimorphism in primates and that male strategies in competition for females and anti-predator defence may be conditional upon this. If this is so, then we have firm evidence that such divergence between female and male energetic strategies was present among early relatives of the Old World monkeys and apes at least 30 million years ago. □

Immunological changes in multiple sclerosis

from Barry R. Bloom

At best one may liken the present state of understanding of multiple sclerosis to that of the blind men and the elephant in the ancient Indian legend. A variety of investigations have each touched a piece of the reality, but no one has been able to integrate the observations into a coherent picture of the disease.

Multiple sclerosis (MS) is an inflammatory demyelinating disease found in a variety of clinical forms, ranging from an exacerbating-remitting course to a chronic progressive disease. Not only is the aetiology unknown, but the variety of clinical patterns and immunological pathological changes seen in different patients are so great that there may be more than a single aetiology — a possibility which provides little comfort to either patients or investigators.

There are two principal hypotheses. The first, based on the induction of inflammatory demyelinating disease in animals immunized with central nervous system tissues or myelin is that MS is an autoimmune disease directed against central nervous system antigens. While the classical animal models of allergic encephalomyelitis (EAE) are not faithful reproductions of the human disease, recent models of relapsing EAE in animals (Raine *et al. Lab. Invest.* 31, 369; 1974; Wisniewski *et al. Ann. Neurol.* 1, 144; 1977) suggest that autoimmune mechanisms could well contribute to the pathology. The difficulty with this hypothesis is that no convincing evidence for autoimmunity to CNS antigens or myelin components has been found in patients with multiple sclerosis.

The second hypothesis is that MS is caused or triggered by a persistent virus infection. This is consistent with the epidemiology of the disease, and with the finding of elevated antibodies to measles and other viruses in the cerebrospinal fluid of MS patients. Exacerbations and

remissions might be explained by emerging antigenic variants, analogous to those seen in the viral demyelinating disease of sheep (Narayan *et al. Science* 197, 376; 1977; Bloom *et al. Neurology* 28, 93; 1978).

Whether triggered by immune reactivity to brain antigens or virus, the histopathologic picture of the demyelinated lesions closely resembles that of cell mediated immune reactions, with lymphocytes and macrophages predominating. However, the often acute onset of symptoms and rapid clinical remissions suggests that mechanisms which are not characteristic of cell mediated reactions, for example, antibodies, immune complexes or edema, are contributing to the disease.

In trying to understand the immunological basis of multiple sclerosis one encounters a frustratingly large number of clues which do not fit into a coherent picture. In particular, there are elevated levels of immunoglobulins, particularly IgG₁, in the cerebral spinal fluid of the majority of MS patients and elevated titers of antibodies are found to a number of viruses, particularly measles. The immunoglobulins found in brain or CSF of MS patients, when subjected to electrophoresis, show a characteristic discrete or 'oligoclonal' pattern of bands. While similar oligoclonal Ig patterns can be observed in samples from patients with several other diseases, (for example, subacute sclerosing panencephalitis (SSPE), viral encephalitis or meningitis, Guillain-Barré Syndrome and neurosyphilis) the banding patterns in MS patients remain constant over long periods while they vary or disappear with time in other diseases. The oligoclonal

electrophoretic patterns of immunoglobulins from CSF or acid eluates of MS brain suggest that they may be derived from a limited number of B-cell clones in the CNS.

In this issue of *Nature*, Mattson *et al.* (p311) report that eluates from individual MS plaques or lesions show limited and discrete patterns of immunoglobulin banding, the sum of which appear to be represented in the pattern of pooled eluates from MS brain. In contrast, electrophoresis of immunoglobulins in SSPE brains shows the same immunoglobulin pattern in different portions of the brain, similar to pooled white matter extracts. Mattson suggests that different immunoglobulin-synthesizing clones are present or expanding in different areas of MS brain, raising the possibility that different antigens may be present in each plaque. In another recent paper, Baird *et al. (J. Immunol.* 124, 2324; 1980) have shown that anti-idiotypic antibodies directed against oligoclonal immunoglobulins in the CSF of one patient with MS crossreacted with immunoglobulins from another, suggesting that two different patients had immunoglobulins with similar, if not identical active sites. The paradox posed by these two results is that different patients may have antibodies directed toward the same antigen, as judged by crossreactive idiotypes; and yet different lesions in the same patients may contain different antigenic determinants, or at least, elicit different antibody clones.

Such sweeping conclusions, however, cannot be accepted without serious reservations. While there may be some cross-reactive idiotypes among immunoglobulins in the CSF of some MS patients, a number of investigations have been unsuccessful in finding crossreactive idiotypes in MS patients. In the case of the discrete electrophoretic patterns derived from different MS plaques, it remains unclear whether the changes in banding are merely quantitative, reflecting, for example, merely different accessibility of old, relative to new lesions to infiltration by B cells, or whether there are real qualitative differences in types of immunoglobulin produced in different plaques. It has been reported by Link and Laurenzi (*Ann. Neurol.* 6, 107; 1979) that individual oligoclonal bands in CSF may contain both κ and λ light chains. This observation, together with the findings of Mattson *et al.* that a number of the bands from different plaques appeared to have common mobility, raises doubts that meaningful conclusions on the number and nature of B-cell clones can be derived from electrophoretic banding patterns of CSF or brain immunoglobulins. An alternative interpretation suggested by Mattson *et al.* is that a mitogenic effect on B-cells of a CNS breakdown product could polyclonally stimulate proliferation of small numbers of B-cells localized in individual plaques to produce 'nonsense'

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antibody. While elevated levels of antiviral antibodies are found in the CSFs of the majority of MS patients, little of the antiviral antibody activity is detected on electrophoresis associated with the oligoclonal bands. It remains unclear to what antigens, if any, the preponderance of the immunoglobulins in MS brains of CSF's are directed.

Another clue that must be considered is the significant genetic association of multiple sclerosis with HLA-D, thought to contain the human counterpart to the murine Ir genes. While the linkage to DW2 is strong among patients in Western Europe and North America, patients in the Middle East and Asia appear to have associations with other HLA-D antigens.

In addition to the relatively unique and stable alterations in immunoglobulin patterns in the CNS of patients with MS, recent observations suggest changes in normal cellular immune parameters as

well. Con A- and virus-induced suppressor activity of lymphocytes from MS patients have been found to be significantly diminished (Antel *et al. Ann. Neurol.* **5**, 338; 1978; Neighbour & Bloom. *Proc. natn. Acad. Sci. U.S.A.* **76**, 476; 1979). Santoli *et al. J. Immunol.* **120**, 1369; 1978). Recently Reinherz *et al. (N. Engl. J. Med.* **303**, 125; 1980) reported a reduction in total T-cells and in T-cell subsets defined by monoclonal antibodies, particularly the T5 subset, in the blood of MS patients during exacerbations. A number of laboratories have recently found reduced capability of lymphocytes from MS patients to produce IFN α (Type I leukocyte interferon) and IFN γ (Type II or 'immune' interferon) (Neighbour *et al. Neurol.* in the press; Riethmuller *et al. 4th Intl. Cong. Immunol.*) and to have reduced natural killer activity (Benczur *et al. Clin. Exper. Immunol.* **39**, 657; 1980; Neighbour *et al. 4th Intl. Cong. Immunol.*). It is interesting

to note that a similar, or even more striking decline in ability to produce interferon or carry out natural cytotoxicity, paralleled by decline in the T5 T-cell subset, is being found in patients with lupus erythematosus, suggesting that these changes are not restricted to multiple sclerosis, but common to several diseases of possible viral aetiology.

It is tempting to infer from these various observations that there is a fundamental defect in immunoregulation in multiple sclerosis. Yet in no instance have the immunological changes been clearly associated with any functional changes in the patients. It is not even known whether the immunoregulatory changes precede attacks of the disease, or whether they follow events, still obscure, which trigger attacks. That there are multiple immunologic changes in MS is clear; but which, if any, are directly related to the disease process remains to be established. $\square$

Quantum mechanics and gravitational waves

from Bridget Marx

It has been said that physics is the science of measurement. The quantum mechanical revolution in the early part of this century threw up many problems and paradoxes concerning measurement and, surprisingly, many of these remain unsolved and little understood. It is true that for the most part physics can progress without a full understanding of the practical and philosophical implications of quantum mechanics but occasionally the full impact of these fundamental problems is felt. The latest group to be left worrying about theories of measurement, post quantum mechanics, comprises those people trying to measure gravitational radiation.

Gravitational waves are predicted by Einstein's theory of general relativity to be ripples in the curvature of spacetime which propagate at the speed of light. What the theoreticians would like the experimentalists to do is, firstly, to demonstrate the existence of these waves, either as a continuous flux or in larger spasmodic bursts, and secondly, to analyse the radiation to give them clues for developing their theories further. However this is no easy task. The effects of gravitational waves are small and not easily detected. Gravitational radiation should be noticeable by its effect on large masses. Experimenters first tried to show its existence by setting up massive antennae and looking for a movement induced by a passing gravitational wave. But the effect predicted by current theory is so small that the sort of displacement one is hoping to measure is of the order of 10^{-19} cm for a resonant bar

with a mass of about one ton. When one considers that the Bohr radius of a hydrogen atom is about 5×10^{-9} cm and the 'classical' electron radius is about 3×10^{-13} cm, then the difficulties of measuring 10^{-19} cm can be appreciated. On top of that the experimenters need to make repeated measurements of the same system to look for changes in its position, so the measurement processes must not be such as to cause unpredictable changes of the position of the system. We are not used to treating large objects quantum mechanically, but it is obvious that it must be done in this case. So what limits does the theory of quantum mechanics impose on this measurement scheme?

The quantum theory of measurement warns experimental physicists that no matter how carefully a measurement is made it will always change the system being measured. Consideration of the problems this causes for the detection of gravitational waves has led to the identification of quantum nondemolition (QND) variables (Braginsky, Vorontsova & Thorne *Science* **209**, 547; 1980; Thorne *Rev. Mod. Phys.* **52**, 285; 1980; Caves, Thorne, Drever, Sandberg & Zimmerman *Rev. Mod. Phys.* **52**, 341; 1980; Caves *Phys. Rev. Letts.* **45**, 75; 1980). A quantum nondemolition variable is one for which a sequence of precise measurements can be made, the result of each measurement being completely predictable from the result of the preceding measurements (Caves *et al.* op cit.) The logic of this can be understood by considering the system of a free electron as discussed by Braginsky *et al.* If we measure the electron's position in space and so force it into a well defined position then the Heisenberg uncertainty principle relating the properties of

position, x , and momentum, p ,

$$\Delta x \Delta p \geq h/2$$

ensures that the momentum is changed by an unpredictable amount by the position measurement. That might not bother us much if we don't want to know the electron's momentum, but it does have an effect if we want to make a second measurement of the position. In the time interval, t , between the measurements the electron has moved an amount $x_t = (p/m)t$ where m is the mass of the electron. But as p is uncertain by Δp then x_t is uncertain by an amount $(\Delta p/m)t$, that is

$$\Delta x_t \geq \frac{ht}{2m\Delta x}$$

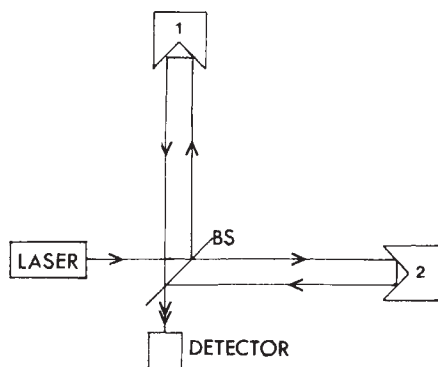
where Δx is the uncertainty of the first measurement. So the more accurate the first measurement the less we can rely on the result of a second measurement of position. Braginsky *et al.* go on to apply the same analysis to a large (10 ton) antenna and show that if the first measurement is made to an accuracy of 10^{-19} cm then a second measurement 10^{-3} second later is uncertain by at least 5×10^{-19} cm. So no subsequent measurement can be compared with the first to the full measurement precision. However, if we had decided to measure momentum rather than position, the second measurement would have been as informative as the first. The position uncertainty would have changed according to the uncertainty principle but this would not have fed back into the momentum unless the electron were in a position-dependent potential. In the situation of a free electron or a freely moving antenna, momentum is a QND observable whereas position is not.

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Generally, most observables are not QND observables and there will be 'back-action' from the measurement. This is a problem which must be overcome if such incredibly accurate measurements are to be made. One solution is to find a QND variable to measure and this is the route proposed for the antenna type of gravitational wave detector. As it is not feasible to make sufficiently precise measurements of momentum another variable must be used. Details of how it is proposed to measure the real and imaginary parts of the complex amplitude of a large quantum mechanical harmonic oscillator, both of which are QND variables, are given by Braginsky *et al.* and by Caves *et al.*

Another suggested method for measuring the displacement of masses produced by gravitational waves, is to use a Michelson interferometer. The basic apparatus is shown in the figure. The laser light enters the interferometer, is split by a beam splitter (BS), passes down the two arms to the mirrors mounted on test masses and is reflected back to the beam splitter where it is recombined. The light measured at the detector is a sinusoidal function of the path difference BS1-BS2, and so is sensitive to any differential displacements of the test masses 1 and 2, such as would be produced by a gravitational wave. As the effect of mirror displacement can be amplified by using multiple reflections in each arm of the interferometer this technique is potentially very sensitive. However, this is a position measuring device and so it is subject to the effects of the Heisenberg uncertainty principle just as is the antenna. The problem considered by Caves and earlier by Edlestein, Hough, Pugh and Martin (*J. Phys. E.* **11**, 710; 1978) is how to estimate the measurement limit imposed on this system by quantum mechanics. There is an uncertainty in measuring the light intensity from the interferometer (remember we are looking for a change in intensity to indicate a differential change in BS1, BS2) arising directly from fluctuations in the laser light source. In addition, differential radiation pressure effects from the light on the mirrors could limit the reliability of measurements, especially at high input intensities. This would imply an optimum intensity for an interferometer device.

Do such differential forces exist in real interferometers? The literature on this point is not convincing. Indeed Caves refers to a 'lively but unpublished controversy' on the subject. The problem is fundamental: radiation pressure is a momentum conserving effect due to the particle nature of the light photons, whereas the interference fringes observed with an interferometer are due to the wave nature of light. The wave-particle duality of light, and indeed all particles, has always caused problems. What happens to a photon in the interferometer? An interesting and instructive discussion of this point is given in an article by Frisch (*Contemp.*



A method for measuring the displacement of masses produced by gravitational waves using a Michelson interferometer.

Phys. **7**, 45; 1965). The important thing to remember is that the problem is very different from the apparently similar experiment of setting up a beam splitter with light detectors in place of the mirrors. In this latter experiment, if a photon were detected at 1 then it certainly wouldn't be detected at 2. So the photon's momentum would be given to either 1 or 2, but not both. However, with the detector placed to monitor the interference pattern — after

the beams are recombined — the only assumption that we can make is that each photon goes down both arms of the interferometer, that is that each photon is reflected from both mirrors. If we knew which mirror the photon had been reflected from, we could not observe an interference pattern. This is an experiment directly comparable with the more famous Young's two-slit experiment (see, for example, Bohm *Quantum Theory* Prentice-Hall, 1951), where if we ask which slit the photon came through we can not observe an interference pattern.

The discussion of the interferometer problem in some of the literature seems to come uncomfortably close to confusing the two-detector and the two-mirror problems.

The whole field of gravitational wave detection is proving to be full of fascinating problems and will no doubt stimulate physicists in all fields of research. It is particularly interesting to note that these attempts to demonstrate the validity of the last great theory of classical physics, that is general relativity, are being limited by effects which are totally due to the new physics of quantum mechanics. □

Galileo saw Neptune in 1612

from David W. Hughes

JOVIAN satellites, lunar mountains, saturnian rings, stars in the Milky Way, Sun spots, phases of Venus — the number of significant observations of astronomical objects made by Galileo Galilei is impressive indeed. The coming together of the invention of the telescope and Galileo's brilliant enquiring mind obviously led to a bountiful harvest. Did Neptune slip through his fingers? Galileo saw the planet but recorded it as a star in his notebook. Was the discovery of Neptune in his grasp, almost 234 years before Johnn Galle found it in 1846? I don't think so. The rudimentary star maps of the time, the inadequate telescope mountings and Neptune's distance from the Sun make it nigh on impossible. But still, like all scientists are taught today, Galileo recorded in his observation journal not only what he was specifically investigating but also unusual sightings and events.

That Galileo actually recorded Neptune was discovered recently by Charles T. Kowal of the Palomar Observatory, California Institute of Technology and Stillman Drake, the Professor of the History of Science of the University of

Toronto. Their findings are reported in this issue of *Nature* on page 311. Prediscovery observations of planets are common and Neptune is no exception, the famous one being by Lalande in 1795. As people were obviously not looking for Neptune in the seventeenth and eighteenth centuries, the only chance of it being accidentally recorded was if it happened to be in close proximity to something that people were looking at. Extrapolating Neptune's orbit back in time revealed that Jupiter, the brightest and most commonly observed planet, actually passed in front of Neptune both in September 1702 and in January 1613. A search of the records of 1702 is a task for the future. Kowal and Drake decided to start by examining Galileo's 1612-1613 observations of Jupiter.

Now Galileo had discovered the four large Jovian satellites Io, Europa, Ganymede and Callisto in January 1610, and had spent much time observing and analysing the orbital movement of this 'miniature solar system'. On 7 September 1612 Galileo put forward a method whereby observations of the satellites of Jupiter from different places on the Earth's globe could lead to a determination of the longitude of that place to an accuracy of about three degrees. This

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would be very useful for the navigation of ships. The idea was to regard Jupiter as a celestial clock whose 'hands' were the four satellites, their positions gave the local time which could then be compared with Florence time (for example) to give the longitude. Galileo was preparing tables to show the satellite positions and observed Jupiter to this end. Kowal and Drake point out that a star near Jupiter was noted on 28 December 1612 and again on 28 January 1613, in a different position, and this star happens to be Neptune. There are no other bright stars in Virgo anywhere near the position of the Jovian-Neptune conjunction of that year. Galileo thought Neptune was a star because it looked just like one in his telescope. The disk of Neptune subtends about 2.4 arc sec at Earth, way below the 1.24 arc min resolution of Galileo's telescope. Two factors can account for Galileo not noticing the movement of Neptune against the fixed star background. First, there was an absence in that area of

the sky of bright stars to form that background and second, the mean daily motion of Neptune is about 22 arc sec, much too small to be detected in those days.

Kowal and Drake's discovery is important because it could lead to an improvement in the accuracy of our knowledge of the orbital parameters of Neptune. Neptune orbits the Sun once every 165 years. As it was only discovered 134 years ago it hasn't been seen to go round once yet. Observations of the orbit over long time periods give clues to the gravitational perturbations the planet suffers and these could lead to the discovery of a planet beyond Pluto. The exact time of the 1702 Jovian occultation of Neptune would be extremely useful in this calculation and this is ample justification for a close scrutiny of the astronomical manuscripts of 1702. Hidden in these might be a record of another historic close encounter between these two planets. □

Laser annealing for semiconductor devices

from I. W. Boyd and J. I. B. Wilson

LASERS are used for cutting, drilling and welding metals and ceramics as well as by surgeons, seismologists and spectroscopists. Now the laser annealing of semiconductors is attracting industrial interest and there have been specialist conferences devoted entirely to this aspect of laser technology^{1,2}. A recent NATO Advanced Study Institute in Italy covered both the intriguing physics of the laser irradiation of semiconductors, and some of the technical advances which will be possible in micro-circuit fabrication.

Annealing is the heat-treatment used to restore disordered semiconductor wafers to crystalline perfection, after selected 'dopant' impurities have been implanted into the wafers (as energetic ions) to adjust the electrical conductivity. Conventional furnace annealing uses temperatures of $\sim 950^\circ\text{C}$ for ~ 30 minutes but has the unfortunate side effect of allowing the dopant atoms to diffuse into the wafer and to spread out laterally. This negates an important advantage of ion-implantation — the high degree of control over the depth and shape of the implanted (doped) region. Each subsequent thermal treatment causes further spreading of dopant and imposes a restraint on the closer packing of devices within an integrated circuit.

Both electron beams and lasers can be used to apply heat locally to semiconductor wafers, with fine spatial and temporal control, so that only part of a wafer may be heat-treated. Lasers, in contrast to electron

beams, have no need for a vacuum enclosure or inert gas cover. The actual temperature reached by a sample will depend both on the laser energy and irradiation time, and on the optical absorption properties of the material and its thermal capacity and conductance.

In silicon, visible laser radiation such as that from a laser ruby (used in the first pulsed laser annealing of ion implanted silicon³), is strongly absorbed whereas infrared CO_2 laser radiation is only weakly absorbed (by the free charge carriers) until the sample heats up (producing many free carriers). Both pulsed and continuous (CW) lasers of various types, can recrystallize disordered silicon, but by different mechanisms.

CW laser annealing is similar to the conventional furnace process, for regrowth of the crystal lattice takes place in the solid phase. The high temperatures are, however, only maintained for milliseconds because the laser beam is scanned across the sample and there is insufficient time for dopant to diffuse into the bulk of the wafer. Extended structural defects (such as stacking faults and dislocations) are more effectively removed, and more of the dopant is placed in electrically-active substitutional sites, than by furnace

treatment. Pulsed lasers are thought to produce a melted region and this allows dopant atoms to migrate over greater distances before the solid/liquid interface moves back towards the surface. Various modes of regrowth are therefore possible for disordered or polycrystalline layers on crystalline substrates, and in some cases surface ripples remain after the laser treatment. Van Vechten⁴ has, however, proposed a theory which places more emphasis on the importance of the electron-hole plasma produced by strong laser pulses. This, he suggests, may sufficiently weaken the covalent bonding of the lattice that rearrangement takes place without formation of a liquid phase. The reflectivity of silicon has been observed to increase sharply during pulsed annealing and although this is generally believed to prove that melting has occurred it is not conclusive. Recent Raman scattering measurements during pulsed annealing of silicon have indicated a lattice temperature of only 300°C (ref. 5), well below the melting point of $\sim 1,400^\circ\text{C}$.

There are, then, two alternative laser annealing modes, which either can leave implanted ions in their original region, or can allow them to redistribute. In fact, novel phases and solid solutions may be formed, which are impossible to produce by conventional furnace treatment. It is also possible to form good ohmic contacts on materials such as gallium arsenide and indium phosphide, the surfaces of which will begin to decompose if they are heated to the usual temperatures required for alloying contacts. Further, it is possible to deposit metal, dielectric, and semiconductor films directly from a gaseous compound on to a substrate by laser. The laser beam may either be used to heat the substrate to a temperature at which the impinging vapour can decompose, or be used to directly dissociate the gaseous molecules.

Reports of actual devices formed by laser processing are much fewer than those devoted to more basic studies of the techniques, but laser annealed p-n junctions and MOSFET's can be made from silicon which was originally in the poly-crystalline state. There are problems associated with the ever-present oxide layer on silicon because it may have anti-reflection properties for the laser light at certain thicknesses, and will have different thermal expansivity from the underlying melted silicon. It is essential to use a laser system without hot spots in the beam, and with accurate beam steering to avoid distortion of the beam shape. New specially-designed equipment is now on the market.

Advanced technology lasers may eventually replace furnaces, which are more costly in energy, for annealing ion-implanted silicon devices, but it is the novel applications which are the most exciting, and which may allow the routine use of new semiconductor materials. □

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ARTICLES

Isotopic evidence for the provenance of some Caledonian granites

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A combined Nd- and Sr-isotope study of Caledonian granites in Scotland and northern England demonstrates the importance of continental crust in their petrogenesis and places limits on the involvement of contemporary mantle-derived material.

THE continental crust is generally thought to have been generated over a considerable portion of Earth history through the progressive chemical differentiation of the mantle. Thus, all igneous, metamorphic and sedimentary rocks residing in the continents are ultimately 'mantle derived'. However, petrologists usually reserve the term 'mantle derived' for those igneous rocks derived from primitive mantle melts by magmatic differentiation processes without an intermediate solid stage. If, after a period of residence in the continental crust, such mantle-derived igneous rocks are remelted, the resultant magmas would be designated 'continent derived'. The provenance of granites is an important aspect of the fundamental problem of crustal generation and growth. Granites that are wholly or partially mantle derived make a net contribution to the mass of the continental crust and are therefore an integral part of crustal growth. In contrast, granites with no mantle-derived component only involve intracontinental differentiation with no concomitant change in continental mass.

To distinguish between mantle and continental derivation in a specific instance, it is necessary to recognize (usually from abundances of radiogenic isotopes) whether or not the precursor material has had a significant residence time in the continental crust. In this context, the main problem has been to find adequate criteria to distinguish between mantle and crustal provenance and in the latter case to quantify the crustal residence time of the precursor material. Hurley and colleagues¹⁻³ pioneered the application of isotope studies to this particular problem using ⁸⁷Sr/⁸⁶Sr ratios. Only recently has the Sm-Nd system been exploited as a tracer of granite provenance^{4,5}. For example, the Tertiary granites of Skye, north-west Scotland, have much lower ¹⁴³Nd/¹⁴⁴Nd ratios than predicted Tertiary mantle values, a feature consistent with a large component of their Nd being derived from Archaean basement^{5,6}. A reconnaissance investigation of Sm-Nd isotope systematics in Phanerozoic granites (including the Sierra Nevada batholith, Himalayan granites and Hercynian granites of France) by Allègre *et al.*⁴ has revealed the presence of continental-derived components in all cases examined.

It must be emphasized that investigations of granite provenance using the isotopic composition of a particular element (such as O, Sr, Nd) only explicitly refers to the provenance of that element whereas the provenance of other constituents can only be inferred. Ideally, therefore, measurements of the isotopic compositions of several elements exhibiting different chemical behaviour during crustal differentiation and evolution are required to make completely general statements about the provenance of a granite as a whole. The present study uses the combined Nd- and Sr-isotope approach for evaluating the

provenance of Caledonian granites occurring in Scotland and northern England and in particular assesses in appropriate situations the possibility of Sm/Nd fractionation attending the differentiation and mobilization of pre-existing continental crust.

Caledonian granite magmatism

The term Caledonian granites encompasses granite (*sensu lato*) rocks that were emplaced into the Caledonides over a period of about 150 Myr, starting ~550 Myr ago and associated with the closure of the Iapetus Ocean^{7,8} (Fig. 1). Two of the oldest of these are the Carn Chuinneag and Ben Vuirich granites, dated, respectively, at 555 ± 10 Myr (ref. 9) and 514 ± 7 Myr (ref. 10) using U-Pb chronology of separated zircons. These belong to Read's¹¹ group of Older granites, and were emplaced before the main Caledonian structural and thermal episodes that occurred ~480-460 Myr ago. The post-tectonic (Newer granites of Read) range in age from ~470 Myr (see ref. 12) to ~390 Myr (refs 12, 13) (Fig. 1). More detailed reviews of the chronology of Caledonian granites can be found elsewhere^{12,14-16}, and geochronological data pertinent to the granites investigated here are summarized in Table 1. The large variation in initial ⁸⁷Sr/⁸⁶Sr ratios of Caledonian granites (see Fig. 1 in ref. 17) is noteworthy. Initial ⁸⁷Sr/⁸⁶Sr ratios ≥ 0.710 are characteristic of the pre-tectonic granites and 475-Myr-old granites of north-east Scotland. It is generally agreed that Proterozoic, or older, crust is involved in their generation^{10,18}, and that they are S-type granites as defined by Chappell and White¹⁹. No such consensus exists for the provenance of the remaining post-tectonic Newer granites that have initial ⁸⁷Sr/⁸⁶Sr ratios ranging down to 0.704.

The U-Pb systematics of Caledonian granite zircons investigated by Pidgeon and coworkers^{9,10,12} are particularly relevant to the problems of granite provenance. Several granites, including both pre-tectonic granites (for example, Carn Chuinneag⁹ and Ben Vuirich¹⁰) and post-tectonic granites north of the Highland Boundary Fault (such as Strichen¹² and Foyers¹²) have been shown to contain zircons with ²⁰⁷Pb/²⁰⁶Pb and U-Pb ages in excess of the age of granite emplacement. These U-Pb data define reverse discordia which have lower intercepts approximating to, or defining, the age of intrusion and upper intercepts corresponding to ages up to at least 1,000 Myr. Such zircons are considered to predate the magmatic event that produced the granites and to have been inherited as refractory constituents from a crustal precursor to the granites^{9,10,12,16}. Therefore, in favourable circumstances they may provide an estimate of the age of some component of the granite.

In addition to studies of radiogenic isotopes, oxygen isotope abundances have been measured by Harmon and Halliday²⁰ in

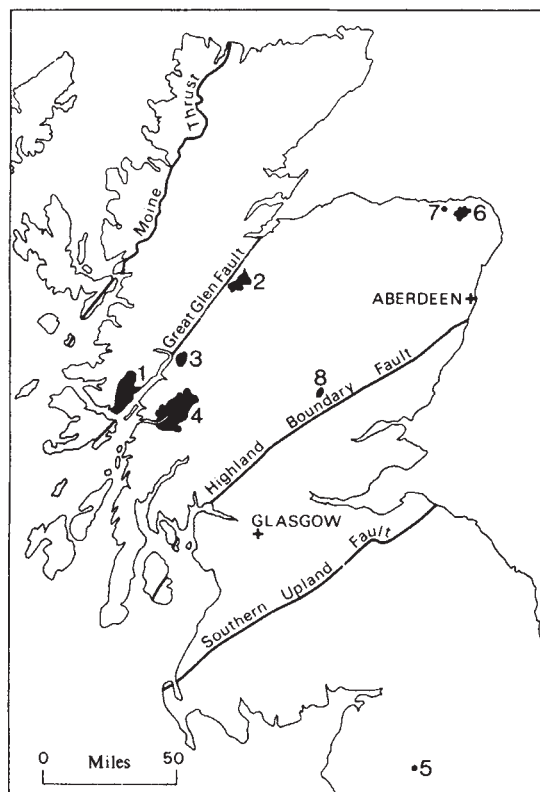


Fig. 1 Map showing the locations of Caledonian granites studied.

1, Strontian; 2, Foyers; 3, Ben Nevis; 4, Etive Complex; 5, Shap; 6, Ben Vuirich; 7, Longmanhill; 8, Strichen.

25 post-tectonic Caledonian granites from both north and south of the Highland Boundary Fault and a more detailed O and Sr isotope study of the Doon, Criffel and Fleet intrusions has also been made²¹. These studies demonstrate the existence of an overall positive correlation between $\delta^{18}\text{O}$ and $^{87}\text{Sr}/^{86}\text{Sr}$, similar to that previously demonstrated for samples from the Peninsular Ranges Batholith²². Harmon and Halliday²⁰ interpret the correlation in terms of a binary mixing process with one component derived from the mantle or new basic lower crust and the other from a Lower Palaeozoic sedimentary source. Thus, granites possessing low $^{87}\text{Sr}/^{86}\text{Sr}$ and $\delta^{18}\text{O}$ values are considered to be dominated by melts derived from mantle source regions with both negligible crustal residence time and minimal contamination with pre-existing sialic crust (see also ref. 23).

The present study of Rb–Sr and Sm–Nd systematics of selected Caledonian granites includes pre- and post-tectonic examples from north of the Highland Boundary Fault in Scotland and the Shap Granite in northern England (see Fig. 1 and Table 1).

Results

Rb–Sr analyses were carried out at the University of Oxford and the Institute of Geological Sciences, London, and Sm–Nd analyses at the Lamont–Doherty Geological Observatory. The analytical methods used have been described previously^{24–27}.

New Rb–Sr results for samples measured in the present study are given in Table 2. Initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios (I_{Sr}) (Table 3) have been calculated assuming emplacement ages given in Table 1. I_{Sr} values for the Strontian tonalites and granodiorites exhibit a relatively small range from 0.70534 to 0.70598, whereas that for the biotite granite phase for Strontian is significantly greater at 0.70713. This agrees well with the I_{Sr} value of 0.70700 ± 14 reported by Halliday *et al.*²¹ for a sample of this same phase. The Foyers samples in Table 3 exhibit a larger range of I_{Sr} values of 0.70436–0.70819 and were considerably more heterogeneous than the Strontian intrusion at the time of emplacement.

These Rb–Sr data, together with those of Halliday *et al.*²¹, are plotted on a Rb–Sr isochron diagram in Fig. 2. The data array for the Foyers granites falls close to the reference line corresponding to the assumed age of emplacement. The remainder of the Foyers' data are also positively correlated but scatter about a line of greater slope. This could reflect mixing between low Rb/Sr–low $^{87}\text{Sr}/^{86}\text{Sr}$ and high Rb/Sr–high $^{87}\text{Sr}/^{86}\text{Sr}$ components or the inheritance of Rb–Sr systematics from the source region which would correspond to an isotopic event at $\sim 1,000$ Myr. Furthermore, the possibility that some of the scatter reflects post-crystallization redistribution of Sr cannot be eliminated at this stage. For Strontian, the Rb–Sr data have been shown to scatter about a line corresponding to an age of 750 Myr (ref. 17), but we note here (Fig. 2) that, relative to a 435-Myr reference line (the emplacement age), it is only the points for the biotite granite which are highly discrepant, perhaps indicating that this unit has a different provenance from the granodiorites and tonalites. It may be relevant that inherited zircon has been reported from the biotite granite phase¹⁶ but not from the other phases in Strontian¹².

Using the emplacement ages given in Table 1, the I_{Sr} values have been computed for the other Caledonian granites studied and are given in Table 3. The total range in I_{Sr} for the granites considered here range from ~ 0.716 (Strichen, Ben Vuirich, Longmanhill) to ~ 0.705 (Ben Nevis, Etive).

$^{147}\text{Sm}/^{144}\text{Nd}$ and $^{143}\text{Nd}/^{144}\text{Nd}$ ratios are presented for 19 samples in Table 3, and initial $^{143}\text{Nd}/^{144}\text{Nd}$ (I_{Nd}) ratios have been computed using their assumed emplacement ages (Table 1). The I_{Nd} values range from 0.511444 (Longmanhill) to 0.512104 (Strontian). Those intrusions with high I_{Sr} values (Ben Vuirich, Longmanhill, Strichen) have among the lowest I_{Nd} values (Table 3), but there is no other detailed correlation. Note also that in the case of the Foyers intrusion the I_{Nd} values are much less variable than the I_{Sr} values.

The I_{Sr} and I_{Nd} values are also compared in terms of Δ parameters²⁸ in Table 3. These parameters express the deviations of isotope compositions with the bulk Earth values at the

Table 1 Geochronological data for some Caledonian granites

Intrusion	Age (Myr)	Method	Comments	Emplacement age (Myr)	Ref.
Strontian	435	U/Pb and $^{207}\text{Pb}/^{206}\text{Pb}$	Nearly concordant zircons from granodiorite		12
	1,462 and 381	U/Pb	Reverse discordia for inherited zircons from biotite granite	435	16
Foyers	Probably predates Lochnagar and Hill of Fare at 415 ± 5 Myr and 413 ± 3 Myr dated by Rb–Sr whole rock. 401 ± 8 Myr K–Ar (biotite)			415	15
Ben nevis	Unpublished Rb–Sr data and 382–399 Myr K–Ar (biotite)			400	15
Etive	Associated with lower old red sandstone lavas				
Shap	390 ± 6	U–Pb	Concordant zircons		12
	394 ± 3	Rb–Sr	Whole rocks and minerals	394	13
	397 ± 7	K–Ar	Micas		13
Strichen	475	U–Pb	Concordant monazite		
	1,000 and 450	U–Pb	Inherited zircon. Lower intercept age may reflect Pb loss	475	12
Longmanhill	Assumed to be the same as Strichen			475	
Ben Vuirich	514 ± 7 and	U–Pb	Reverse discordia for inherited zircon	514	10
	$1,316 \pm 26$				

Table 2 Rb-Sr data for Caledonian granites measured in the present study

Sample and rock type		Rb (p.p.m.)	Sr (p.p.m.)	$^{87}\text{Rb}/^{86}\text{Sr}$	$^{87}\text{Rb}/^{86}\text{Sr}^*$	Sample and rock type		Rb (p.p.m.)	Sr (p.p.m.)	$^{87}\text{Rb}/^{86}\text{Sr}$	$^{87}\text{Rb}/^{86}\text{Sr}^*$
Foyers						Strontian					
PM16	Tonalite	62	1,385	0.1288	0.70682 (5)	ϕ 203	Tonalite	70	1,223	0.1647	0.70700 (5)
PM28	Tonalite	55	1,284	0.1233	0.70691 (4)	ϕ 212	Tonalite	51	1,100	0.1334	0.70652 (5)
PM18	Granodiorite	62	1,070	0.1668	0.70791 (6)	ϕ 200	Tonalite	74	1,036	0.2056	0.70687 (5)
PM21	Granodiorite	73	938	0.2240	0.70765 (5)	ϕ 202	Tonalite	68	877	0.2232	0.70686 (5)
PM20	Granodiorite	65	900	0.2079	0.70748 (5)	ϕ 201	Granodiorite	58	1,186	0.1407	0.70621 (4)
PM23	Granodiorite	61	837	0.2098	0.70773 (4)	ϕ 207	Granodiorite	59	1,172	0.1447	0.70624 (4)
PM19	Granodiorite	70	810	0.2487	0.70764 (5)	ϕ 204	Granodiorite	63	1,084	0.1673	0.70630 (5)
PM22	Granodiorite	71	685	0.2982	0.70996 (4)	ϕ 205	Granodiorite	80	790	0.2916	0.70764 (5)
PM17b	Granodiorite	60	646	0.2673	0.70865 (7)	ϕ 209	Granodiorite	81	752	0.3100	0.70758 (5)
PM27	Granite	73	378	0.5558	0.70765 (5)	ϕ 210	Granodiorite	86	628	0.3940	0.70795 (7)
PM24a	Granite	81	350	0.6661	0.70871 (5)	ϕ 208	Biotite granite	92	613	0.4317	0.70980 (5)
PM26b	Granite	76	332	0.6589	0.70845 (6)	Etive Complex					
PM25	Granite	75	328	0.6580	0.70848 (4)	Starav					
PM26a	Granite	85	300	0.8155	0.70943 (6)	A ϕ 51	Granite	110	497	0.6402	0.70907 (5)
PM24b	Granite	91	273	0.9595	0.71026 (6)	A ϕ 52	Granite	121	739	0.4727	0.70772 (6)
Ben Nevis						Cruachan					
P73-3	Granite	95	635	0.4306	0.70701 (7)	A ϕ 59	Adamellite	128	509	0.7278	0.70897 (5)
Shap											
CCR8	Granite	266	435	1.769	0.71740 (8)						

* Numbers in parentheses refer to the last digit and is the 2σ error.

appropriate times of emplacement. In Fig. 3 the Δ values are compared with the bulk Earth, for which $\Delta_{\text{Sr}} = \Delta_{\text{Nd}} = 0$ at all times. Depleted mantle sources, such as represented by mid-ocean ridge basalts (MORB) or the 505-Myr Bay of Islands ophiolite²⁹, have negative Δ_{Sr} and positive Δ_{Nd} values. In contrast, the continental crust, which is the complement to depleted mantle, has a mean composition with a positive Δ_{Sr} and negative Δ_{Nd} values, although both positive and negative Δ_{Sr} values are known to exist in high grade metamorphic terrains^{5,6}. None of the granites analysed as part of this study have Δ values characteristic of depleted mantle; on the contrary, all have negative Δ_{Nd} and positive Δ_{Sr} , similar to much of the continental

crust. In this respect, these data are entirely analogous to those reported by Allègre *et al.*⁴, and indicate a crustal contribution to all these granite bodies.

The Sm-Nd isotope systematics of Archaean meta-igneous rocks^{6,27,30-35} can often be satisfied by simple two-stage models. The first stage can be considered to start some 4,550 Myr ago and end with a crust-mantle differentiation event at t ($t \geq 2,500$ Myr). Isotopic evolution in this first stage is characterized by a Sm/Nd ratio indistinguishable from the average chondrite ratio. The second stage extends from t until the present ($t = 0$). This comparatively simple model often seems contradictory to geological considerations that require more complex multistage

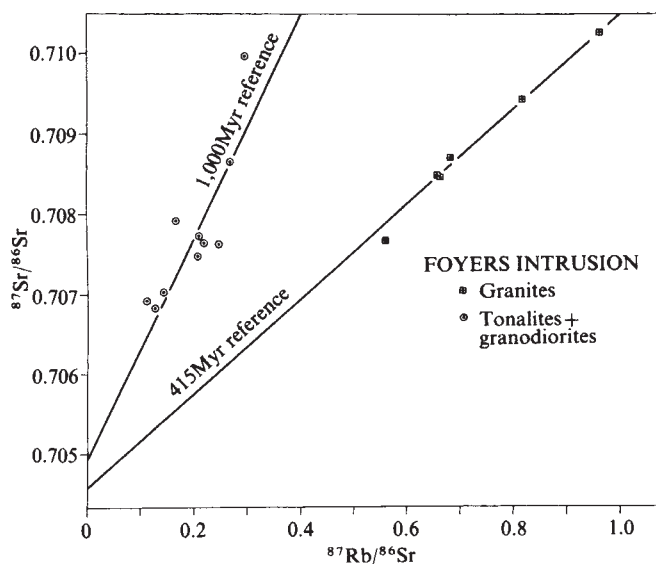
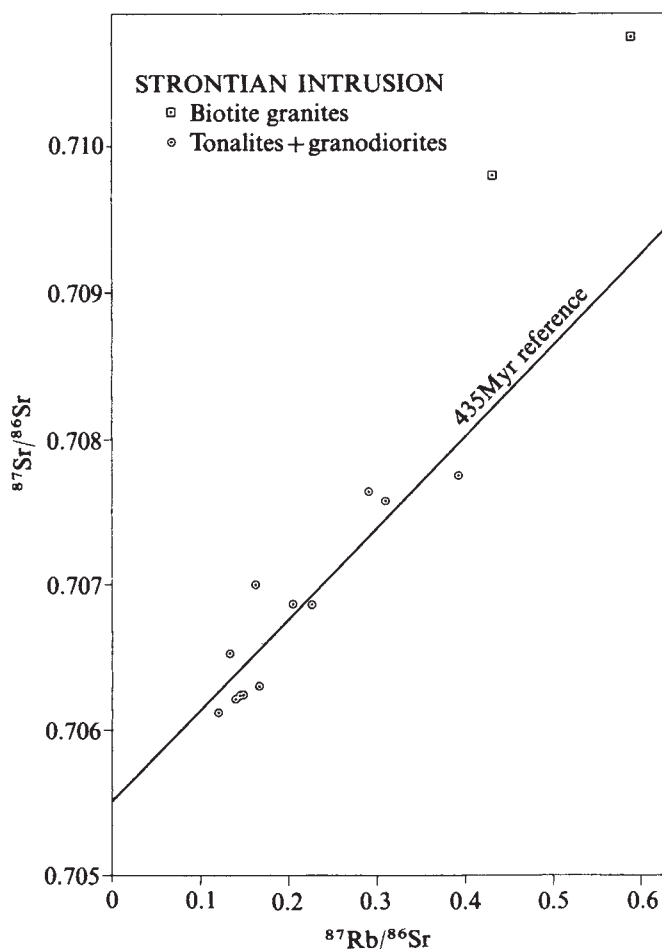


Fig. 2 Rb-Sr isochron diagrams for granite, granodiorite and tonalite phases of the Foyers and Strontian intrusions (Table 2 and ref. 16). The Foyers granites plot close to a 415-Myr reference line (assumed time of emplacement), and the tonalites and granodiorites plot close to a 1,000-Myr reference line. The Strontian tonalites and granodiorites plot close to a 435-Myr reference line (assumed time of emplacement), but the biotite granites do not.



development but merely indicates that the differentiation processes responsible for the production of such rocks occurred over an interval Δt that is not readily resolved from the Sm–Nd isotope systematics.

Model ages (t_{CHUR})³⁰ have been calculated (Table 3) for each sample assuming such a two-stage evolution. There is no *a priori* reason to assume that these ages should correspond to specific geological events. However, available data indicate that the Sm/Nd fractionation associated with the production of a new crustal segment is likely to be most significant in the history of a given crustal segment, and furthermore suggest that intracrustal differentiation processes involving erosion, sedimentation and metamorphism may have comparatively little effect on Sm/Nd ratios^{36–40}. Thus, in some instances t_{CHUR} may provide a reasonable estimate of the time of separation of the Sm and Nd in their precursors from the mantle. t_{CHUR} can be expected to be a minimum age for this event if the mantle reservoir had been previously depleted and had a higher $^{143}\text{Nd}/^{144}\text{Nd}$ ratio than the bulk Earth, or if granitic melt production within the continental crust involved a fractionation of Sm/Nd. Furthermore, if a component derived from the continental crust is mixed with a contemporary mantle-derived component, the t_{CHUR} will not correspond to the provenance age of either component and will, depending on the precise nature of the mixing, usually be a minimum age for the crustal component.

Having noted these limitations, t_{CHUR} model ages range from close to the time of granite emplacement (for example, Shap) to ages much in excess of the emplacement ages (for example, 1,500 Myr for Strichen).

Provenance of Caledonian granites

The presence of inherited zircons and high initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios have demonstrated that the early Caledonian granites must contain a large component derived from the continental crust^{9,10,12,16}. The Sm–Nd isotopic results reported here for the Ben Vuirich, Strichen and Longmanhill granites are entirely consistent with this viewpoint: the Δ_{Sr} and Δ_{Nd} values are very different from those typical of depleted or undepleted mantle, and indicate the existence of a large crustal component. One possible crustal component is the 2,920-Myr (ref. 6) Lewisian basement, which is known from north of the Highland Boundary Fault. Comparison of the Sm–Nd systematics of the Lewisian gneisses⁶ with those of these early granites shows that they are

not dominated by a Lewisian component. This fact is reflected by the t_{CHUR} ages which, although in some cases considerably greater than the assumed emplacement age, are much younger than the Lewisian. In contrast, the t_{CHUR} ages of the Tertiary Skye granites, which average 2,400 Myr, are considered to contain a large component derived from the Lewisian⁵. U–Pb data reported for inherited zircons in the Ben Vuirich granite define a discordia that intercepts the concordia at $1,316 \pm 26$ Myr and 514 ± 5 Myr. The upper intercept age is in excellent agreement with the t_{CHUR} age of $1,310 \pm 70$ Myr. This limits any 'mantle-derived' component in the granite to a negligible proportion and further indicates minimal fractionation of Sm/Nd during remelting of this Proterozoic crustal source. Although less well constrained from zircon data, the same argument may apply in principle to the petrogenesis of the ~475-Myr granites of north-east Scotland.

The origin of the remaining post-tectonic (Newer) granites is more controversial. For example, distribution of inherited zircons ($\geq 1,000$ Myr old) led Pidgeon and Aftalion¹² to propose a major Proterozoic crustal component in granites north of the Highland Boundary Fault. Caledonian granites south of the fault were ascribed to a Lower Palaeozoic crustal source. In contrast, many other workers^{20,23,39–41} have invoked a major involvement, either directly or indirectly, of contemporary (Caledonian) mantle-derived material, with crustal contaminants ranging from Lewisian³⁹ to Lower Palaeozoic in age.

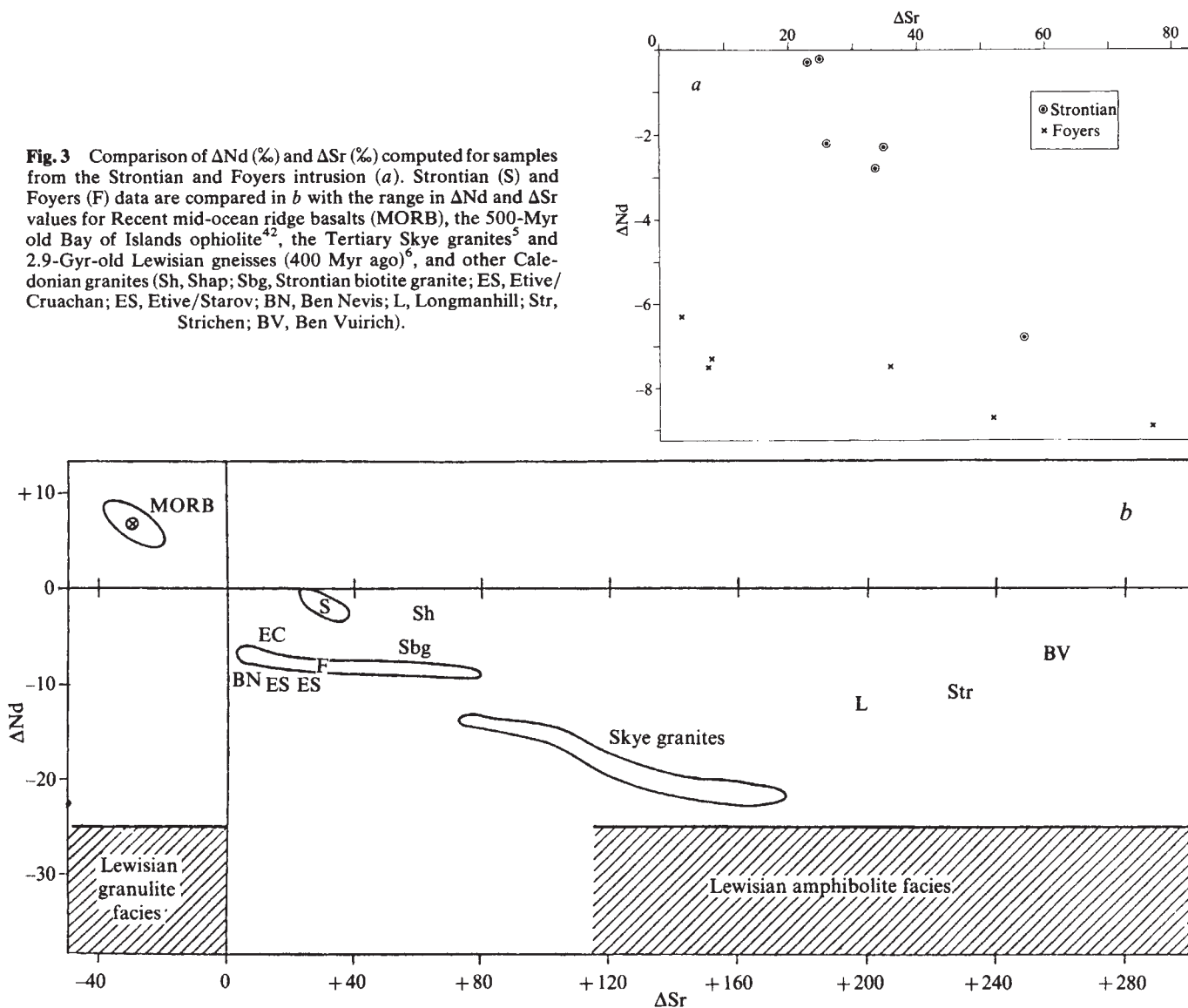
The Sm–Nd systematics of the Newer Caledonian granites provide some resolution of these possibilities. The Δ_{Nd} and Δ_{Sr} values are compared in Fig. 3b with the expected values of depleted mantle source regions in the Caledonian (as represented by the field of mid-ocean ridge basalts computed for 400 Myr ago and by the 505-Myr-old Bay of Islands ophiolite) and the Lewisian gneisses, these being the most extreme compositions likely to be involved. Other potential components, such as Proterozoic or Lower Palaeozoic crustal sources, will be intermediate in isotopic composition. The main question is whether the Δ_{Nd} and Δ_{Sr} values of the granites plotted reflect mixtures between such extreme end-member compositions or whether they are generated from younger continental crust. It is obvious that none of the granites are dominated by Nd and Sr derived from a contemporary depleted mantle source, and even in the case of Strontian granodiorites and tonalites a contribution from

Table 3 Sm–Nd data and initial strontium ratios for some Caledonian granites

Intrusion	Sample	Sm (p.p.m.)	Nd (p.p.m.)	$^{147}\text{Sm}/^{144}\text{Nd}$	$^{143}\text{Nd}/^{144}\text{Nd}$ ($\pm 2\sigma$)	I_{Sr}	I_{Nd}	ΔNd	ΔSr	$t_{\text{CHUR}}(\text{Myr})$	f^*
Strontian	$\phi 202$	4.065	26.00	0.09398	0.512372 (30)	0.70548	0.512104	−0.2 (6)	25 (1)	440 (50)	−0.51
	$\phi 203$	4.795	27.74	0.1040	0.512292 (30)	0.70598	0.511996	−2.3 (6)	35 (1)	630 (50)	−0.46
	$\phi 205$	5.231	32.99	0.09537	0.512240 (30)	0.70583	0.511968	−2.8 (6)	32 (1)	650 (50)	−0.51
	$\phi 207$	6.524	32.06	0.1224	0.512450 (34)	0.70534	0.512101	−0.3 (6)	23 (1)	450 (70)	−0.37
	$\phi 210$	3.730	19.93	0.1125	0.512320 (24)	0.70550	0.511999	−2.2 (4)	26 (1)	640 (40)	−0.42
	$\phi 208$	3.112	18.01	0.1039	0.512050 (32)	0.70713	0.511754	−6.8 (6)	57 (1)	1,040 (50)	−0.46
	Foyers	PM16	9.927	59.90	0.09963	0.512009 (30)	0.70605	0.511738	−7.5 (5)	36 (1)	1,060 (50)
PM18		12.50	72.05	0.1043	0.511963 (24)	0.70692	0.511680	−8.7 (4)	52 (1)	1,190 (40)	−0.46
PM22		4.482	34.04	0.07919	0.511883 (29)	0.70819	0.511668	−8.9 (5)	77 (1)	1,030 (40)	−0.59
PM24b		1.769	12.69	0.08394	0.511971 (22)	0.70458	0.511743	−7.5 (4)	7.6 (6)	960 (30)	−0.57
PM26a		2.416	16.71	0.08695	0.511993 (31)	0.70461	0.511757	−7.3 (6)	8.0 (7)	950 (40)	−0.55
PM27		1.760	13.06	0.08105	0.512026 (26)	0.70436	0.511806	−6.3 (5)	3.3 (6)	860 (30)	−0.58
Ben Nevis		P73–3	3.874	26.51	0.08785	0.511964 (22)	0.70455	0.511734	−8.0 (4)	6.5 (10)	1,000 (30)
Etive Complex	Starav	A051	2.890	0.08941	0.511870 (30)	0.70542	0.511636	−9.8 (6)	23 (1)	1,160 (40)	−0.54
	Starav	A052	3.762	0.09536	0.511868 (26)	0.70503	0.511618	−10 (1)	16 (1)	1,230 (40)	−0.51
Cruachan	A059	4.851	30.44	0.09585	0.512124 (30)	0.70482	0.511873	−5.4 (6)	12 (1)	840 (40)	−0.50
Shap	CCR8	6.525	43.20	0.09080	0.512261 (30)	0.70750	0.512027	−2.6 (6)	63 (1)	590 (40)	−0.54
Strichen	P71–17A	8.035	44.67	0.1082	0.511821 (16)	0.7161	0.511484	−11 (1)	ca. 230	1,490 (30)	−0.44
Longmanhill	P71–16A	7.474	37.89	0.1186	0.511813 (30)	0.7143	0.511444	−12 (1)	ca. 200	1,720 (60)	−0.39
Ben Vuirich	BV–1	8.538	38.70	0.1326	0.512137 (32)	0.7174	0.511691	−6.3 (6)	ca. 260	1,310 (70)	−0.32

* f is the deviation of measured Sm/Nd ratios from the bulk Earth (or CHUR) value³¹.

Fig. 3 Comparison of ΔNd (‰) and ΔSr (‰) computed for samples from the Strontian and Foyers intrusion (*a*). Strontian (S) and Foyers (F) data are compared in *b* with the range in ΔNd and ΔSr values for Recent mid-ocean ridge basalts (MORB), the 500-Myr old Bay of Islands ophiolite⁴², the Tertiary Skye granites⁵ and 2.9-Gyr-old Lewisian gneisses (400 Myr ago)⁶, and other Caledonian granites (Sh, Shap; Sbg, Strontian biotite granite; ES, Etive/Cruachan; ES, Etive/Starov; BN, Ben Nevis; L, Longmanhill; Str, Strichen; BV, Ben Vuirich).



this could not exceed $\sim 10\%$, although a larger contribution from an undepleted (that is, $\Delta Nd \approx \Delta Sr \approx 0$) source would be permissible. Similarly, the contamination of mantle-derived material with an Archaean crustal source (such as Lewisian) must be restricted to a maximum of $\sim 30\%$ even in the most favourable case (Foyers). However, the trend of ΔNd and ΔSr values for the Foyers intrusion (Fig. 3a) is incompatible with a simple mixing between mantle and Lewisian endmembers. Rather, the range of Δ values indicates intracontinental differentiation processes, such as sedimentary fractionation of Rb/Sr at relatively constant Sm/Nd, in which case the t_{CHUR} ages (800–1,200 Myr) may give a reasonable estimate of provenance. The broad agreement between the average t_{CHUR} age and the inherited Rb–Sr systematics supports this interpretation, as does the presence of $> 1,000$ -Myr-old inherited zircon. For the Strontian intrusion, possible mantle- and continent-derived components are less readily constrained, but the similarity of the ΔNd and ΔSr values of the biotite granite to the Foyers samples, together with the evidence for inherited zircon, may indicate a similar Proterozoic crustal provenance at least for this phase.

The Sm–Nd and Rb–Sr data also provide constraints on the provenance of post-tectonic granites that do not contain inherited zircon. The t_{CHUR} ages for the Ben Nevis and Etive granites of $\sim 1,000$ Myr are similar to those of the Foyers granite, indicating that their provenance is dominated by Precambrian crust rather than a contemporary mantle source, as has usually been inferred from Sr-isotope data alone. Note, in this respect, that the comparatively low I_{Sr} value for Ben Nevis of

0.7043 in ref. 25 is associated with a $\delta^{18}O$ value of 7.8‰, which is at the lower end of the range of $\delta^{18}O$ values reported by Harmon and Halliday for Caledonian granites²⁰. This combination, as interpreted by these authors, reflects a major mantle or new lower crustal component ($\sim 70\%$). However, this conclusion is at variance with the constraint imposed by the t_{CHUR} age of $\sim 1,000$ Myr. The interpretation of the I_{Sr} – $\delta^{18}O$ correlation for late Caledonian granites as the result of mixing of two components is certainly an oversimplification⁴² and low $\delta^{18}O$ and low $^{87}Sr/^{86}Sr$ are not adequate criteria for the recognition of a granite dominated by a contemporary mantle-derived component.

The lack of inherited zircon in the Shap granite¹² and the t_{CHUR} age of 560 Myr suggest that its provenance is not dominated by an old crustal component. It is possible that this intrusion is derived from a contemporary mantle-derived component or from crustal components that have had comparatively short crustal residence times. The Sm–Nd data alone do not favour either of these alternatives, but taken together, the $\delta^{18}O$ value of 11‰ and $I_{Sr} = 0.07075$ (ref. 20) suggest a sedimentary rather than a mantle provenance. The tonalites and granodiorites of the Strontian intrusion have both low I_{Sr} values (~ 0.7056) and low $\delta^{18}O$ ($\sim 7.2\%$)²⁰. These parameters, coupled with the t_{CHUR} ages of 400–600 Myr, are quite compatible with a provenance involving a large mantle-derived component. However, in view of the Ben Nevis data, it is not possible to exclude a provenance dominated by a crustal source, albeit one with a comparatively short crustal residence time.

Conclusions

In terms of more general questions of crustal evolution, it is important to ascertain the extent to which granite magmatism (s.l.) reflects intracrustal differentiation in the continents *vis-à-vis* the addition of mantle-derived material to the continents. The results of the present study and previously published isotopic data suggest only minimal, if any, involvement of mantle-derived Sr and Nd components for many of the analysed intrusions. Thus, their formation has involved the recycling of continental crustal materials that have had a prior crustal residence time of ≥ 500 Myr (such as Strichen, Ben Vuirich, Ben Nevis, Etive and Foyers intrusions and the Strontian biotite granite). The Rb–Sr and Sm–Nd isotope data reported here do not directly constrain the provenance of other constituents of the granites present in major, minor or trace proportions: mixing processes can be envisaged whereby the provenance of various elements may indeed differ. However, where indications of provenance using different approaches involving, for example, zircon U–Pb, Rb–Sr and Sm–Nd whole-rock systematics concur (as for Ben Vuirich, Strichen, Foyers), it is more reasonable to assume that all constituents of the granite have the same provenance. Although the isotope systematics of these Caledonian

granites indicate a dominance of crustal provenance, it cannot be inferred that the heat required for the melting process was also crustal: this could equally well be supplied from the mantle.

For the remaining intrusions studied there is no unequivocal support for involvement of mantle-derived material that has not had some prior crustal residence. In the case of the Strontian tonalites and granodiorites, involvement of crust with only ~ 100 – 200 Myr prior residence time is difficult to distinguish from the possibilities or alternatively or additionally involved mantle components. In some cases (such as the Shap granite) a short crustal residence of the precursors is indicated by the comparatively high I_{Sr} and $\delta^{18}\text{O}$ values. However, in the case of the Ben Nevis and Etive granites, their t_{CHUR} ages of 800–1,200 Myr suggest that the well documented positive correlation of $\delta^{18}\text{O}$ and I_{Sr} for the Caledonian granites may not be simply attributed to mixing of local upper-crustal sediment with a contemporary mantle-derived component.

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Deformation of the Indo–Australian plate

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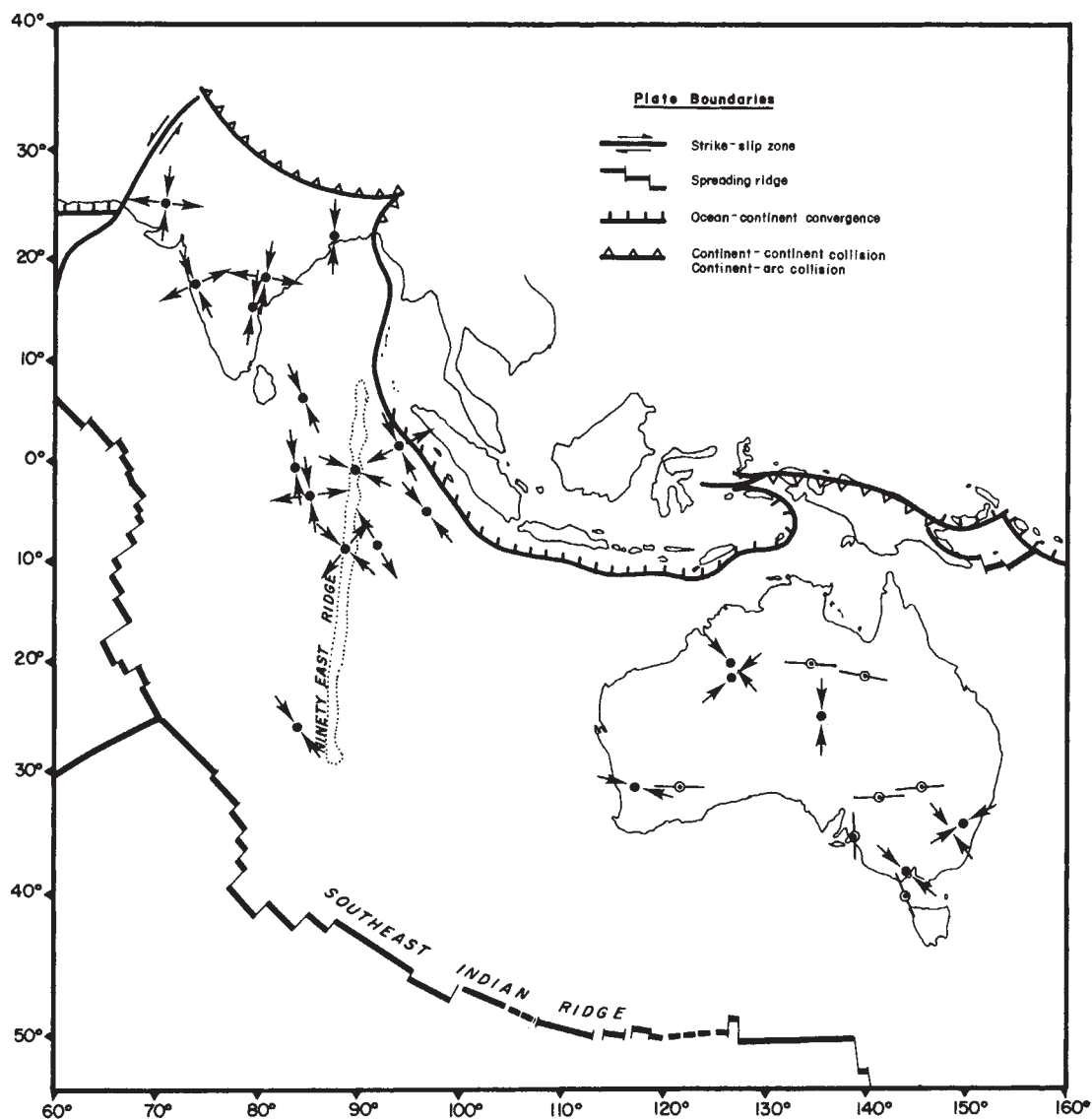
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Intraplate seismicity, deformed oceanic crust and sediments, and local areas of abnormally high heat flow characterize significant internal deformation of the Indo–Australian plate in the northeastern Indian Ocean. The deformation appears analogous to the buckling of an elastic plate with brittle failure at its surface. It is not clear whether the Indo–Australian plate can be resolved into two component parts or whether a new convergent plate boundary is likely to form in the deformed region. The onset of intraplate deformation in the late Miocene seems to be connected to the Himalayan orogenic stage of the collision between India and Asia.

CENTRAL to the concept of plate tectonics are the assumptions that a strong lithosphere overlies a weaker asthenosphere, and that lithospheric plates are undeformed except at their boundaries. Because the evolution of the Earth's surface from the standpoint of plate tectonics has gained widespread acceptance, observations that apparently violate its basic assumptions should be closely examined. Intraplate earthquakes, marine

seismic reflection profiling, and heat flow observations delineate an outstanding example of deformation remote from the boundaries of the Indo–Australian plate (which carries both India and Australia, Fig. 1). This article (1) discusses these observations in detail; (2) points to a possible link between deformation in the interior of the Indo–Australian plate and the Himalayan orogeny; (3) speculates on the implications of treat-

Fig. 1 Directions of principal stress axes for intraplate earthquakes within the Indo-Australian plate²⁻⁸. Inward-pointing arrows denote the axis of maximum compression; outward-pointing arrows refer to the axis of minimum compression. Also shown (—○) are maximum compressional stress directions from *in situ* stress determinations in Australia⁹.



ing the deformation as buckling of an elastic plate; and (4) considers whether the intraplate deformation is a precursor of a new convergent plate boundary within oceanic lithosphere of the Indian Ocean.

Intraplate earthquakes and contemporary stress

Seismologists have long noted that the northern part of the Indo-Australian plate displays a high level of intraplate earthquake activity¹⁻⁴. Shallow events occur over a wide band extending from the Indian peninsula through the Australian continent¹⁻³ and principal stress directions^{2,4,5-8} determined for the most significant of these earthquakes are shown in Figs 1 and 2. Except for the single tensional event east of the Ninetyeast Ridge⁴ (Fig. 2), principal stress directions and *in situ* stress determinations indicate that the northern part of the Indo-Australian plate is under predominantly horizontal compression. The orientations of the axes of maximum compression change from about N-S in the Indian peninsula to nearly E-W in the Australian continent, a feature predicted by global models in which intraplate stress is determined by assumed plate boundary forces¹⁰.

The significant teleseismicity in this supposedly mid-plate region led Sykes² to propose that a new convergent plate boundary may be in the process of forming between Sri Lanka and northwestern Australia. Sykes argued that a new plate boundary would accommodate plate motions that have been

resisted by the continuing collision between continental India and Asia. However, Stein and Okal⁴ questioned the nascent island arc hypothesis of Sykes². They noted an apparent association between the Ninetyeast Ridge north of about 10° S and several large earthquakes which have occurred since the early part of the century. Stein and Okal⁴ proposed that the northern part of the Ninetyeast Ridge is either a plate boundary characterized by left lateral strike slip motion, or that it is the focus of earthquake activity in an area of highly unusual mid-plate tectonics. In either event, the intraplate earthquakes and *in situ* stress measurements (Figs 1 and 2) show clearly that horizontal compression currently characterizes a major portion of the Indo-Australian plate.

Deformation structures

Direct evidence for deformation within the oceanic part of the Indo-Australian plate was found by Eittreim and Ewing¹¹ from a seismic reflection survey of a small area at the distal end of the Bengal Fan (Fig. 2). The reflection records revealed folding of the sediments and high-angle faulting through to basement along an azimuth of ~100°. Further north in the thicker sedimentary section of the Bay of Bengal, Curray and Moore¹² and Moore *et al.*¹³ recognized deformed sediments beneath a prominent unconformity and refracting surface that they could trace throughout the Bay. These previous studies showed that tectonic disturbance of originally flat-lying sediments should be easily seen on seismic reflection records with typical vertical

exaggerations of 25:1. With this in mind, we examined available seismic records from the northeastern sector of the Indian Ocean (held by Lamont-Doherty Geological Observatory and the National Solar-Terrestrial Geophysical Data Center in Boulder, Colorado) to delineate the geographical extent of permanent deformation within the oceanic portion of the Indo-Australian plate, and to determine the types of structures produced at the surface of oceanic lithosphere deformed under horizontal compression. Clear evidence for intraplate deformation is observed only in seismic reflection records from the southern portions of the Bengal and Nicobar Fans, where deformation of oceanic crust and overlying sediments can be resolved into two parts based on dominant 'wavelengths'.

On a broad scale (100–300 km), acoustic basement (probably oceanic crust) is deformed into 'undulations' with 1–3 km of relief (Fig. 3). Large amplitude (30–80 mGal) free-air gravity anomalies are clearly associated with the broad basement features. Gravity anomalies of such amplitudes and wavelengths will arise if the observed undulations reflect uniform bending of the entire oceanic crust⁸. Sea surface heights measured from numerous GEOS 3 orbital paths across the northern Indian Ocean also reflect the broad-scale basement features interpreted from seismic reflection data and marine gravity data (Fig. 3), although small geoidal undulations with wavelengths 100–300 km are less accurately determined by satellite altimetry than by marine gravimetry¹⁴.

On a smaller scale (5–20 km), seismic reflection data from the southern part of the Bengal Fan and the Nicobar Fan¹⁵ reveal

high-angle faulting of acoustic basement (oceanic crust) and overlying sediments, and folding of the sedimentary section. (Note, for example, the faulted basement high 200 km from the left side of Fig. 3 and the general disturbed nature of the sediment cover on this profile.) Two seismic reflection profiles from the southern part of the Bengal Fan, which show typical characteristics of the smaller-scale deformation west of the Ninetyeast Ridge, are shown in Fig. 4.

Of particular importance are the high-angle faults which disrupt the oceanic crust and most of the overlying sedimentary section (Fig. 4a, b). The involvement of oceanic crust in the faulting precludes movement along a surface of decollement above basement as a cause of the deformation. Spatially, the short-wavelength structures are concentrated at 'deformation fronts' where high-angle faults are particularly closely spaced (see Fig. 4a). These deformation fronts, which often form barriers to the southward movement of turbidites over the fan, are separated by less disrupted terrane particularly west of about 86° E.

The seismic profile shown in Fig. 4b, which is located between 86° E and the Ninetyeast Ridge, is interesting because it is located very close to the epicentre determined for a $M_s = 6.1$ (ref. 8) strike slip earthquake⁵ (Fig. 2). Near the epicentre, high-angle faults which disrupt acoustic basement with throws of the order of hundreds of metres cause little offset of the seafloor (Fig. 4b). In fact, 3.5 kHz echosounder data show that these faults offset the sea floor and immediate sub-sea floor reflectors by only 9–18 m. Offsets that increase with depth suggest that the

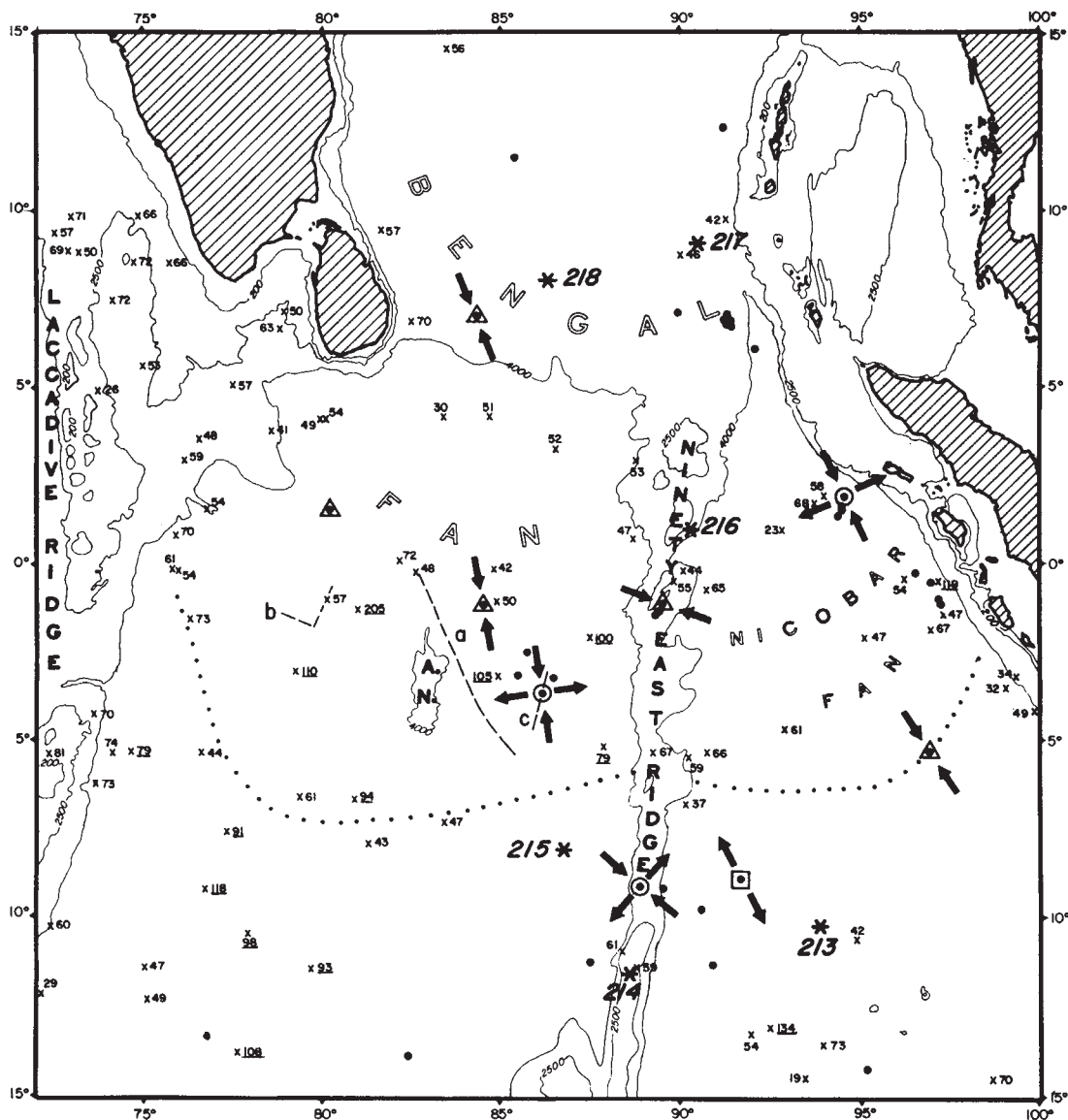


Fig. 2 Directions of principal stress axes for intraplate earthquakes²⁻⁸ and heat flow values^{20,34} in mW m^{-2} (numbered crosses) in the northern Central Indian Ocean. [Note that $41.87 \text{ mW m}^{-2} = 1 \text{ HFU} (\mu\text{cal cm}^{-2} \text{ s}^{-1})$]. ●, Intraplate earthquakes. △, Focal mechanisms with thrust faulting; ○, with strike slip faulting; □, normal faulting⁴. Unexpectedly high heat flow values are underlined. *, DSDP sites. The southern limit of fan deposits is shown roughly by the dotted line. Dashed lines locate seismic reflection profiles shown in Figs 3 and 4. AN, Afanazy Nikitin sea-mount group.

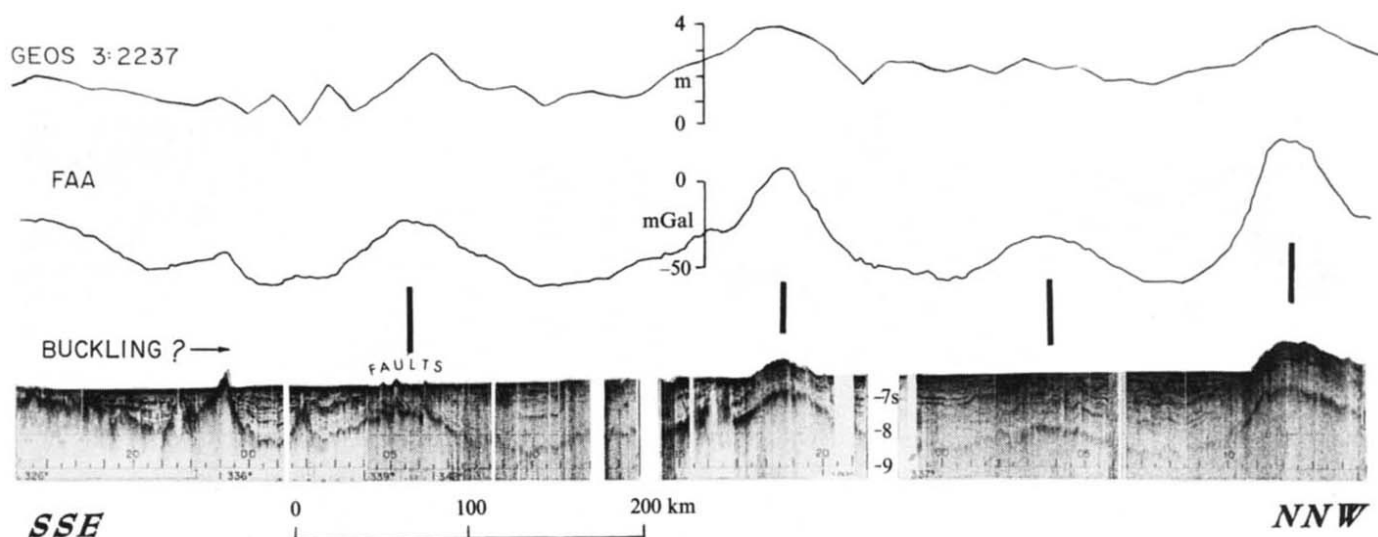


Fig. 3 Broad-scale deformation of the oceanic crust beneath the southern portion of the Bengal Fan (profile a in Fig. 2). Large-amplitude free-air gravity anomalies (FAA) and small-amplitude geoidal undulations (GEOS 3:2237) correlate with the basement deformation.

faults may be growth structures. The small scale structures show a preferred structural asymmetry; the north side is generally upthrown on the high-angle faults and the asymmetric folds, which are likely caused by drag on faults, are generally southward-verging.

Sediments below a prominent unconformity show geometrically conformable structures while above this unconformity the intensity of deformation decreases towards the sea floor (Fig. 4a, b). At DSDP Site 218, which is further north in the Bay of Bengal (Fig. 2), a widespread unconformity and refracting surface separating locally deformed strata from overlying fan turbidites was found to be uppermost Miocene in age^{13,16}. It is likely that this is the major unconformity observed in the seismic reflection records as all Lamont-Doherty piston cores which penetrated the section beneath the unconformity over the southern part of the Bengal Fan contained uppermost Miocene or older faunal groups (ref. 17 and G. Blechschmidt, personal communication). We suggest, from drilling and coring results and the seismic reflection data, that intraplate deformation began in late Miocene time, and that this event is recorded by a prominent unconformity in the sedimentary section of the Bengal Fan.

Both the broad- and small-scale structures provide convincing evidence for deformation remote from the generally accepted boundaries of the Indo-Australian plate. Figure 5 is a map of the broad- and small-scale structures based primarily on single-channel seismic reflection data and gravity data. Several points should be noted. First, the trends of the basement 'undulations' change from ENE-WSW near the Ninetyeast Ridge to E-W or even WNW-ESE in the southwestern part of the Bengal Fan. Second, the basement undulations show culminations and saddles along their trends. Third, the broad structures appear disrupted by the major fracture zone at 86° E which offsets E-W trending magnetic lineations left-laterally by about 850 km (ref. 18). Acoustic basement is shallower on the eastern side of this fracture zone by about 1 km. Fourth, between the 86° E fracture zone and the Ninetyeast Ridge, the trends of the small-scale folds and faults may differ from the trends of the broad basement undulations (Fig. 5). Given the difficulty of tracing individual faults or folds from track to track with the available data distribution, however, this last point should be regarded as tentative.

The available seismic reflection records show that sediment and upper crustal deformation extends from the southern part of the Bengal Fan across the Ninetyeast Ridge into the northwestern Wharton Basin. Compared with the structures observed in the Bengal Fan, deformation of Nicobar Fan sediments in the Wharton Basin appears less severe¹⁵, but seismic reflection

coverage is more extensive west of the Ninetyeast Ridge. Seismic profiles over the Ninetyeast Ridge reveal high-angle faulting¹⁹, although the crust of the Ninetyeast Ridge is not as extensively disrupted as the oceanic crust beneath the Bengal Fan. This is unexpected considering the apparent association of relatively large-magnitude earthquakes and the northern portion of the Ninetyeast Ridge⁴, and we return to this point below.

Heat flow

The third geophysical parameter which reflects significant tectonic activity within the Indo-Australian plate is heat flow. Presently available geothermal measurements in the northern part of the Indian Ocean (Fig. 2) are mainly random values obtained with the Ewing thermograd (Lamont-Doherty Geological Observatory), or with the Bullard probe (Scripps Institution of Oceanography)²⁰. Most heat flow observations lie within the 50–60 mW m⁻² range predicted for the late Cretaceous through early Eocene crust in the northeastern Indian Ocean¹⁸ by the cooling half-space model for oceanic lithosphere²¹. However, several measurements (that are judged reliable²⁰) within the region of deformation delineated by seismic reflection, and some apparently outside that region, yield heat flow values considerably higher than expected (underlined values in Fig. 2). The mean of these high values (107 ± 36 mW m⁻²) is appropriate for heat flow in lithosphere much younger than the early Eocene-late Cretaceous age established for this region from magnetic lineations¹⁸. The occurrence of abnormally high values scattered amongst normal values in a mid-plate region is indeed unusual; these are the only excessively high values in old lithosphere in the entire Indian Ocean²⁰.

Measurement of anomalous heat flow in the oceans is not unusual in itself. Active hydrothermal convection of seawater affects fully one-third of the ocean floor²²; values lower than the predicted heat loss are expected in such geothermal regimes, as unmeasured heat is being carried from the sea floor by convecting fluids. However, in areas with a thick, uniform sedimentary cover (such as the areas covered by fan deposits in Fig. 2), heat loss by convection is unlikely to occur^{23,24}. To explain abnormally high values scattered amongst expected values, local heat sources in addition to the normal heat flux from the lithosphere must contribute to these values. We believe that the additional heat required is associated with the intraplate deformation and we speculate that heat generated by friction on faults in the crust during earthquakes might be involved. If this is the case, there should be a close spatial correlation between the most

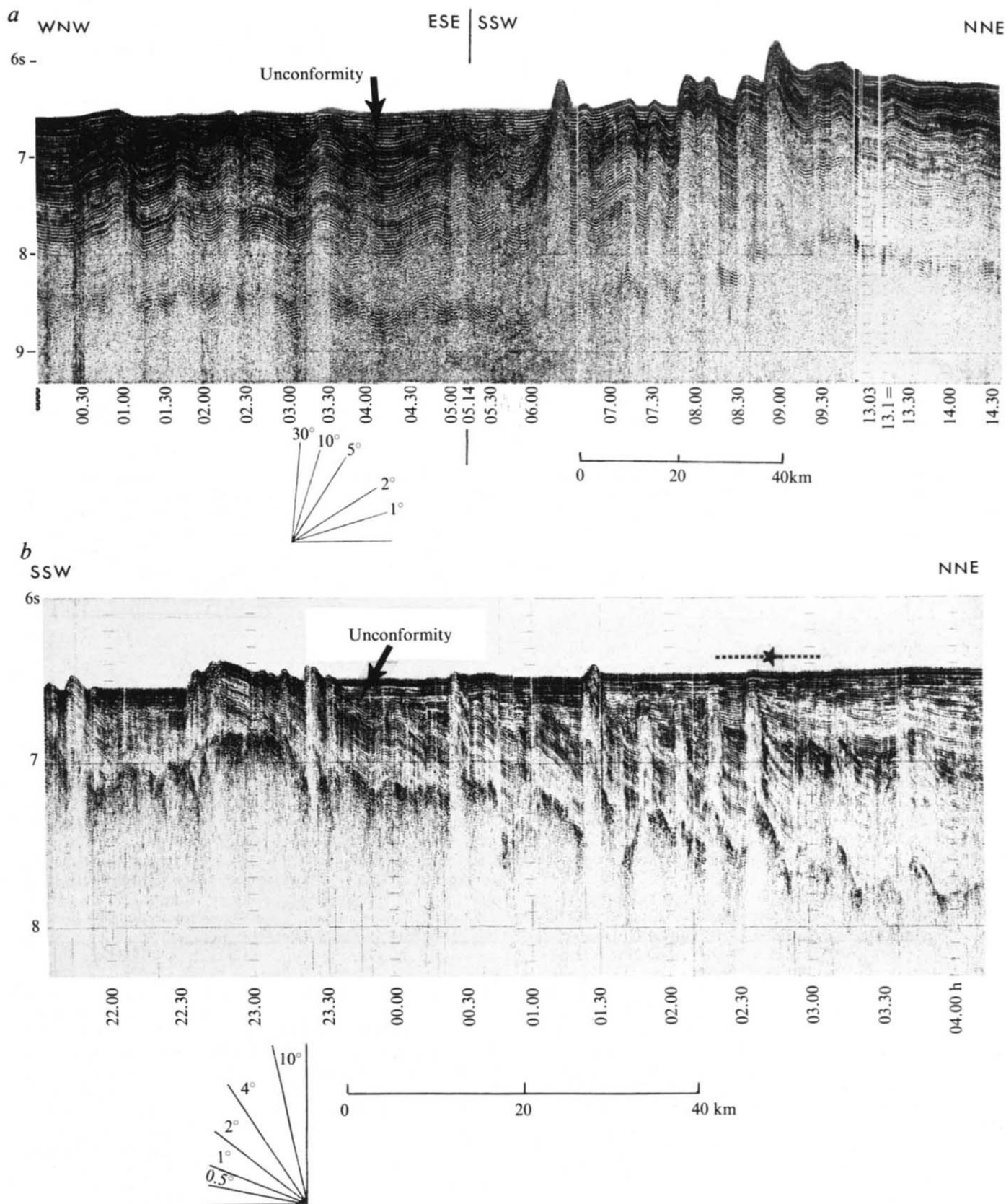
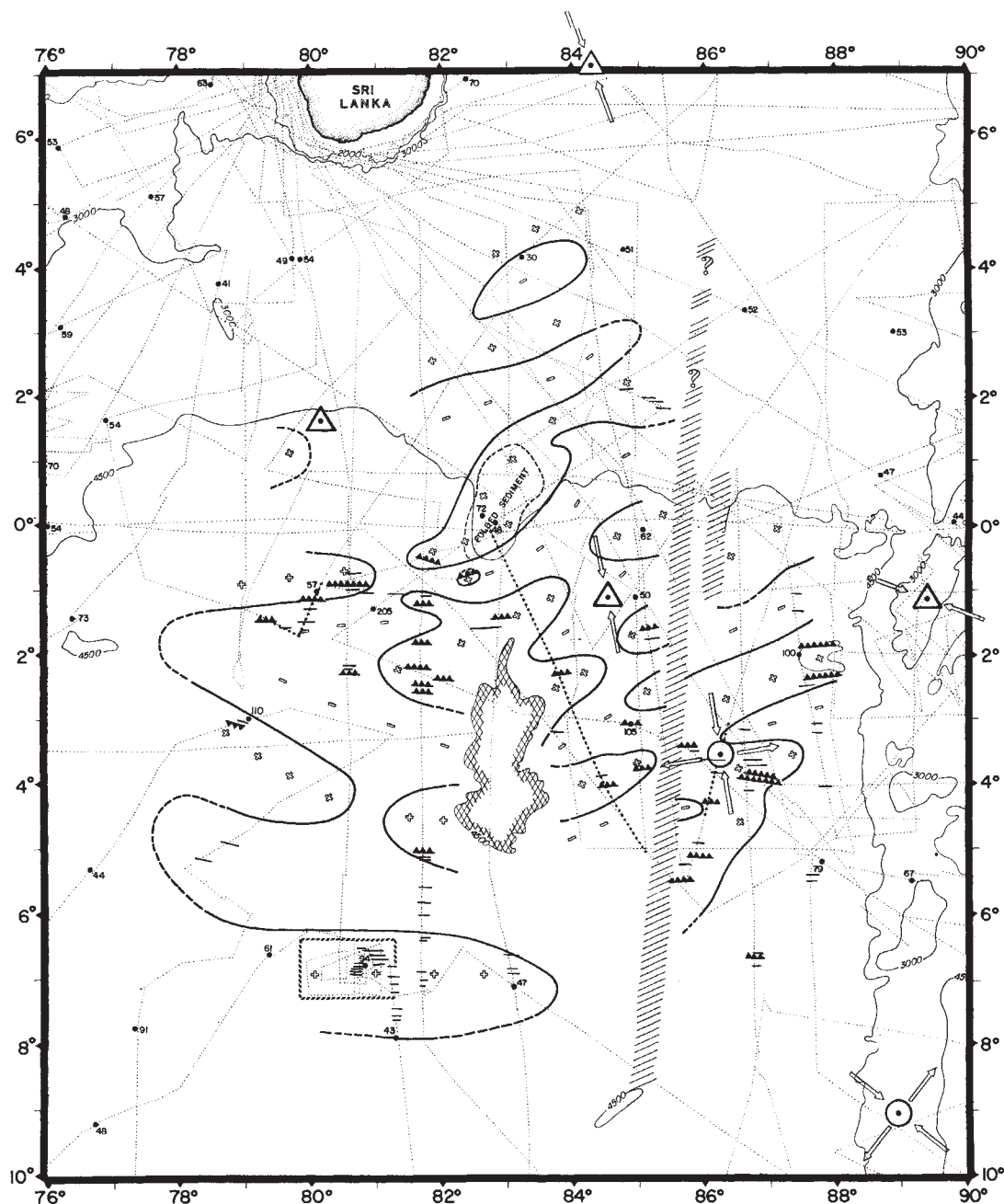


Fig. 4 *a*, Single-channel seismic reflection profile which crosses a prominent deformation front in the southwestern portion of the Bengal Fan (profile *b* in Fig. 2). Note the course change near the middle of the profile. On the WNW-ESE track segment, sediment structures have wavelengths that are long compared to those for structures along the SSW-NNE track segment, indicating an approximate E-W structural grain in the area of this profile. The major unconformity marked by the arrow is probably uppermost Miocene in age (refs 13, 16, 17 and G. Blechschmidt, personal communication). VE ~ 20:1. *b*, Single-channel seismic reflection profile that passes very close to the epicentre of a strike slip intraplate earthquake (profile *c* in Fig. 2) denoted by star and broken line. The major uppermost Miocene unconformity is particularly clear on this profile. Note the difference in fault offsets of basement and of the sea floor in the vicinity of the epicentre. VE ~ 22:1.

Fig. 5 Broad-scale basement structures are outlined by the heavy lines. Plus signs denote relative highs and minus signs relative lows. Saw-tooth symbols denote outcrop of major high-angle faults with the saw teeth on the upthrown side. Minor high-angle faults are the lighter lines. Numbered dots are heat flow values^{20,34} taken from Fig. 2. Principal stress directions for intraplate earthquakes are also from Fig. 2. A major fracture zone at about 85° E with 850 km of left-lateral offset¹⁸ is denoted by the single hatching. The Afanazy Nikitin seamount group is outlined by cross-hatching. Ships' tracks are shown by light dotted lines while the heavy portions denote profiles shown in Figs 2–4. The boxed area at 7° S, 80° E is the survey area of Eittreim and Ewing¹¹.



tectonically disturbed areas and abnormally high heat flow values. This speculation remains to be tested because available heat flow measurements are too widely spaced (Fig. 2).

Limitations of the data

Because seismicity, seismic reflection data and heat flow define regions of intraplate activity of different geographic extents, an examination of the limitations and special characteristics of these three geophysical parameters is warranted here.

The use of single-channel seismic reflection data to map the geographic extent of deformed oceanic crust and the types of structures produced by tectonic stress is limited by several factors. First, the sound sources used to acquire most of the data available to us were too small to penetrate completely through the thick sedimentary section in the Bay of Bengal. Second, while seismic records with large vertical exaggerations provide a good qualitative picture of the structures developed in response to tectonic stress, a quantitative determination of the dips of the observed high-angle faults is not obtainable. Thus, whether the faults are normal or reverse cannot yet be determined. Third, although the deformation of the Indo-Australian plate recorded in seismic reflection data from the southern part of the Bengal

Fan is undoubtedly important, it is possible that other regions of the plate have undergone significant deformation which is not readily apparent in single-channel seismic data. It must be realized that thick stratified sediments provide ideal conditions for examining deformation structures acoustically whereas the same amount of crustal deformation might be difficult to detect in areas devoid of sediment cover or in areas with a thin acoustically transparent pelagic drape.

The time window resolved by each parameter is another factor to be borne in mind when comparing the geographic extents of deformation indicated by earthquake seismology, heat flow, and seismic reflection. Earthquakes have been recorded only for the most recent fraction of geological time. Yet, earthquakes reveal stress information from continental as well as oceanic areas (Fig. 1). Abnormally high heat flow values observed today (Fig. 2) may reflect movements in the crust that occurred long ago compared to the time span of earthquake seismology. Assuming that these anomalous values are expressions at the sea floor of frictional heat generated at the surface of the crust by intraplate earthquakes, we can determine the time taken for conduction of a frictional heat pulse to the sea floor. For a sediment column 1-km thick and an assumed thermal

diffusivity of $10^{-2} \text{ cm}^2 \text{ s}^{-1}$, the peak value of anomalous heat flow reaches the sea floor $\geq 10^5 \text{ yr}$ after generation of the heat pulse in the oceanic crust²⁵. The longest time window is resolved by seismic reflection data because seismic profiles portray the entire history of sedimentation and intraplate deformation in the interior of the Indo-Australian plate. As noted above, the disruption of post-Miocene sediments decreases towards the sea floor (Fig. 4a, b), so that at seismic sound source frequencies, deformation of sediments which have been deposited during the time spanned by historical seismology is not detectable. However, offsets of the sea floor and immediate sub-sea floor reflectors of several metres can be resolved using high frequency (3.5 kHz) sound sources.

Discussion

The evidence presented above for the internal deformation of the Indo-Australian plate allows us to develop a working hypothesis to explain these observations. Several aspects of this have been touched on by previous workers^{2-4,11,26}.

Intraplate compression capable of causing widespread deformation within the Indo-Australian plate probably arose in late Miocene time, according to DSDP and piston core evidence. Assuming that intraplate stress is sensitive to forces applied at plate boundaries and to the base of the plates¹⁰, we speculate that a major tectonic event could have effected changes in forces acting on the Indo-Australian plate in Miocene time, triggering the observed intraplate deformation. The inception and growth of the Himalayan chain in post-Oligocene time has been cited as a significant change in the collisional tectonics between India and Asia²⁶⁻²⁸. From initial contact in the Eocene until early Miocene, deformation was accommodated along a contact zone between India and Asia, whereas intra-continental thrusting within the Indian block has occurred since the early Miocene²⁷. If the second or Himalayan stage of collision between India and Asia greatly increased resistive forces along the plate edge and these were transmitted to the interior of the plate as large horizontal compression, a late Miocene onset of intraplate deformation might reasonably be expected. As suggested originally by Curray and Moore¹², the sedimentation and deformation histories of the deep sea fans of the northern Indian Ocean likely reflect major tectonic events in the collision between India and Asia.

Whatever the history of the intraplate compression, its magnitude has been sufficient to deform oceanic lithosphere into broad undulations (Fig. 3), and produce brittle failure in the oceanic crust (Fig. 4a, b) over a large area in the northern Indian Ocean (Fig. 5). The general appearance of the deformation is that of a buckled plate (Fig. 3). Assuming that a thin elastic plate of Young's modulus $6.5 \times 10^{11} \text{ dyn cm}^{-2}$ is buckled under horizontal compression into a wavelength of 160 km, we determine an apparent elastic thickness for the plate of 12 km and a buckling stress²⁹ of 24 kbar. However, experiments in rock mechanics indicate that yielding by fracture occurs near the surface of the plate and deformation by flow occurs deep within the lithosphere before such high stresses determined from elastic models are attained^{30,31}. The fact that brittle deformation is widely observed in seismic profiles over the region of intraplate deformation suggests that the upper part of the plate has been exposed to stresses in the kbar range. An interesting outcome of the buckling calculation is the anomalously thin effective elastic thickness implied by the wavelength of the undulations. Observations of lithospheric flexure in the oceans have shown that the effective elastic thickness increases with the age of the lithosphere at the time of loading³². At the onset of intraplate

deformation in late Miocene time, the oceanic lithosphere beneath the southern Bengal Fan would normally have an effective elastic thickness³² of 20–30 km. This is greater by a factor of ~ 2 than the 12 km obtained by treating the intraplate deformation as a buckling effect. Assuming that the elastic plate model applies to the real Earth, this discrepancy may indicate either that since the interior of the plate came under large horizontal compression several million years ago the original elastic 'core' of the oceanic lithosphere has been reduced in thickness by yielding at the top and bottom of the plate, or oceanic lithosphere responds to uniform horizontal compression in a way that is different to its response to surface loads.

The large region of permanent strain inferred from seismic reflection data suggests that if the Indo-Australian plate consists of two distinct plates at present, convergence between these plates may be accommodated in a complicated, non-uniform way over a zone several hundred kilometres wide rather than along an obvious plate boundary. This point is important to plate kinematics considerations, as Minster and Jordan³³ found that by treating the Indo-Australian plate as two plates, they could overcome systematic misfits between their model for present-day plate motions (which included a single Indo-Australian plate), and observed Indian Ocean spreading rates and directions. They showed that the observed rates and directions around the Indian Ocean are consistent with about 1 cm yr^{-1} of NW-SE convergence between an 'Indian' plate and an 'Australian' plate along the northern part of the Ninetyeast Ridge³³. An order of magnitude calculation by Stein and Okal⁴ using seismic moments of large events on the Ninetyeast Ridge, produced left lateral slip at a rate of 2.2 cm yr^{-1} along the ridge, consistent with the convergence rate determined by Minster and Jordan³³. Although both sets of calculations have limitations, the general agreement between them suggests, at first glance, that the northern part of the Ninetyeast Ridge is a resolvable strike-slip plate boundary between the two parts of the Indo-Australian plate. Aside from the question of continuity of this plate boundary, the slip rate along the Ninetyeast Ridge determined by Stein and Okal⁴ would relieve all strains predicted from the calculation of Minster and Jordan³³ without any internal deformation of the lithosphere away from the Ninetyeast Ridge. The observations discussed here show that all tectonic displacement between an 'Australian' and an 'Indian' plate is not accommodated by slip along the northern part of the Ninetyeast Ridge, even though strains accumulated through intraplate deformation away from the Ridge are not determinable from the available data.

Finally, do the observations, particularly the seismic reflection data, indicate the impending formation of an intra-oceanic lithosphere subduction zone as suggested by Sykes²? While both the broad undulations and the brittle deformation observed in seismic records (Figs 3 and 4) can be attributed to large horizontal compressions within the plate, fracturing of the crust occurs on high-angle faults and not on shallow-angle thrusts that might herald the beginnings of subduction. During our examination of the available reflection data from the north-east Indian Ocean, we found only one example (near 4° S ; 87° E) of possible shallow underthrusting (from south to north) throughout the entire region of intraplate deformation within the Indo-Australian plate. We feel that this question cannot be answered at present.

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Actin in the inner ear: the remarkable structure of the stereocilium

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The actin filament bundle in the stereocilium of the cochlear hair cell has liquid transverse order, but paracrystalline axial order. A single bonding rule, which determines how a cross bridging protein links neighbouring filaments, accounts for this unusual structure. Such liquid order is possible for bundles of other filamentous proteins.

CRYSTALLINE order consists of chemically identical subunits arranged in identical environments. In such an arrangement, we can conclude that the bonding rule for any one subunit is the same as that for all other subunits, even though the details of the atomic interactions between subunits may be unknown. In biological structures the exact equivalence of crystalline bonds is quite often relaxed to a condition of quasi-equivalence¹. In a quasi-equivalent structure, chemically identical subunits are deformed slightly so that bonds can be formed between subunits which are in almost but not exactly identical environments. In such a structure all subunits make essentially the same specific contacts and thus can be described by a single bonding rule. The concept of the specific bond, which leads to the equivalent or quasi-equivalent arrangement of subunits, has been the basis for understanding the organization of macromolecules in a number of protein-containing structures such as the spherical and rod-shaped viruses, microtubules, and actin filaments.

Biologists, however, do not often find the regular order seen in the examples above. In micrographs of many cellular structures such as the terminal web in the cells of the intestinal brush border², the organization is accompanied by great disorder. Compared to such disordered structures, the bundle of actin in the stereocilium of the inner ear of the alligator lizard appears highly ordered. Yet, to the protein crystallographer, who is accustomed to the near perfect regularity of crystals, the most striking feature of this bundle would be its disorder. The stereocilium is an organizational intermediate between the regular order of the icosahedral viruses and the irregular order of the terminal web. It is interesting because it gives us insight into the organization of partially disordered structures.

The stereocilia or hairs of the hair cells, which extend outward from the apical surface, are elongated processes responsible for the transduction of sound; that is, motion of the stereocilia caused by sound produces changes in the membrane potential of the hair cell. Structural studies of the hair cells in the cochlea of the alligator lizard^{3,4} show that each hair cell contains about 75

stereocilia of varying size with the largest having a length of 30 μm and a diameter of 0.8 μm . Within each stereocilium is a large polar bundle⁵ of more than 3,000 actin filaments which shows an irregular liquid order in transverse section⁶ (Fig. 1a). Surprisingly some transverse sections show a periodic motif we call festooning, which starts at one edge of the stereocilium and continues to the other (Fig. 1b). How can such long-range lateral periodicity arise in a structure with no discernible lateral order? The answer lies in the helical symmetry of the actin and provides insights into the structural principles governing actin bundles and bundles of helical filaments in general.

What is festooning?

The motif seen in the images consists of scalloped rows of actin filaments rather like the folds in drapes pulled to the side of a window. The scalloped rows lie one on top of the other to create the festooned motif. The separation between rows is of the order of 100 $\text{\AA}$ and seems to be constant in all stereocilia. In contrast, the period of the festooning is large, being of the order of 1,000 $\text{\AA}$. In a single section cut through all 75 of the stereocilia that extend from a single hair cell, the direction and period of festooning are generally constant from one stereocilium to the next, but often different for stereocilia from neighbouring hair cells. In an occasional hair cell, however, the stereocilia are disturbed so that, instead of all being exactly parallel to each other, they extend at different angles. In such cells both the period and the direction of the festooning vary considerably. For example, in Fig. 1c stereocilium A has a period of 2,400 $\text{\AA}$ and in a neighbouring stereocilium, B, the direction of the festooning is rotated nearly 90° and the period is 960 $\text{\AA}$.

What gives rise to festooning? If the motif simply and directly reflects a corresponding structural organization of the linked actin filaments, then different patterns of festooning would have to correspond to different structural organizations within the stereocilia. Alternatively, we propose that all stereocilia have a single common structure and the different motifs arise from

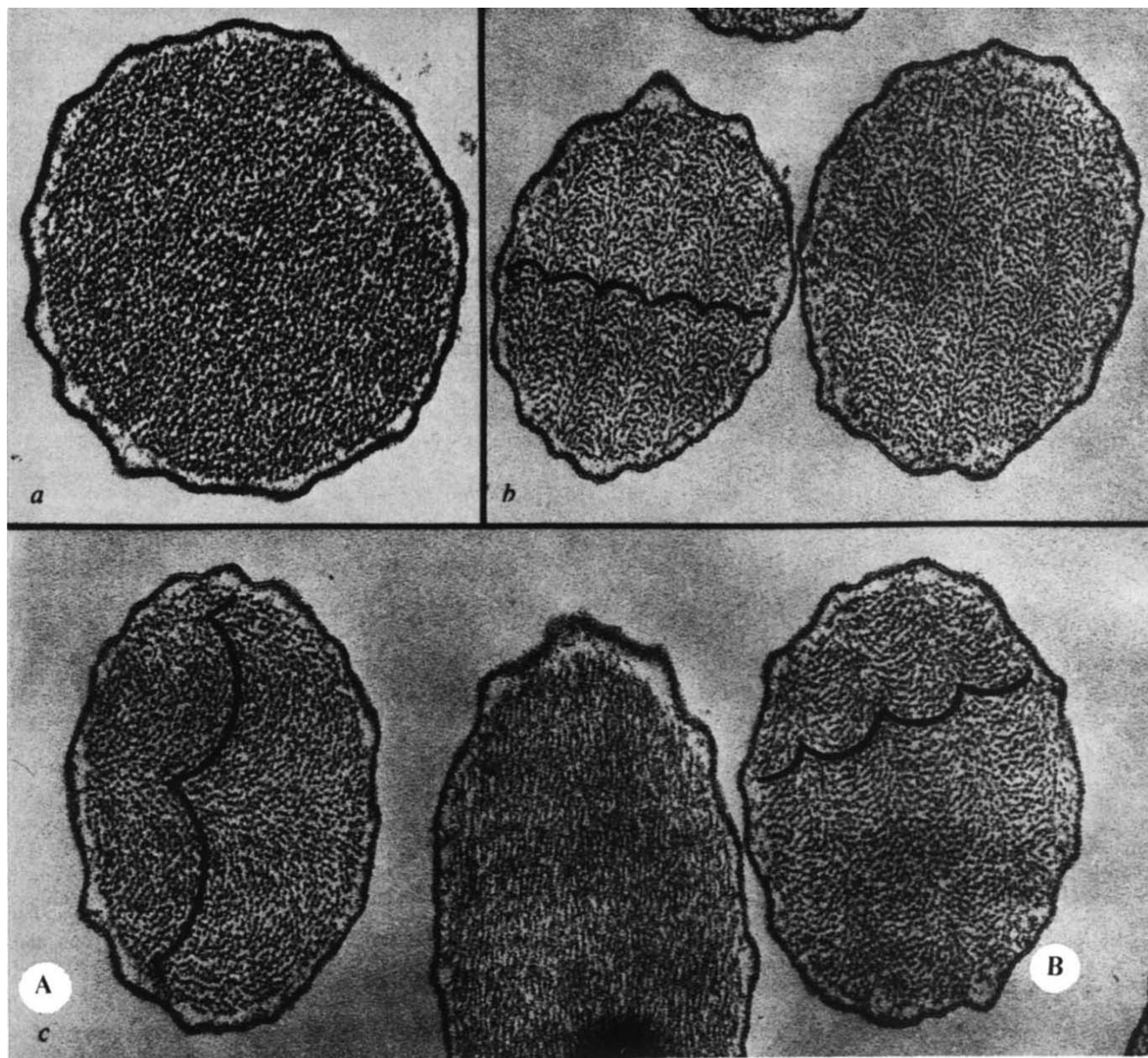


Fig. 1 *a*, Thick transverse section (375 Å or thicker) of a stereocilium from the cochlea of the alligator lizard. Thick sections give no hint of a long-range spatial periodicity but show a liquid arrangement of filaments having a mean interfilament spacing of 100 Å. Optical diffraction patterns of such sections confirm the liquid order showing a pattern consisting of a single diffuse ring. *b*, Thin transverse section of two stereocilia. This section, which is of the order of 200 Å thick, shows a scalloped or festooned pattern with a period of 680 Å. Note that the period and direction of the pattern are the same in both stereocilia. *c*, Thin transverse section of a group of stereocilia. In this cell the orderly and parallel arrangement of stereocilia has been disturbed. As a result the period and direction of festooning are different for different stereocilia. The period in stereocilium A is 2,400 Å and that in B is 960 Å. All magnifications are 120,000.

different orientations of the stereocilia relative to the plane of section. Many features of festooning are explicable as a consequence of taking a thin oblique section through layers of parallel rods, where each successive layer is rotated with respect to the previous layer. This was observed by Bouligand *et al.* for arrangements of chromatin fibres⁷. In the same way, if the crossbridges linking filaments are helically arranged, the axial pattern of crossbridges will be converted into a transverse motif by a slight tilting of the plane of section off a true transverse direction. This is similar to Reedy's discussion⁸ of thin sections of insect flight muscle in which a motif having a long period is seen to arise from the periodic crossbridging of myosin to actin. If this explanation is correct for the stereocilium, the period of festooning should depend on the angle of the section relative to the axis of the stereocilium and the direction of the festooning on

the direction of the tilt of the stereocilium relative to the knife in the microtome. Differences in the images of different stereocilia then would not indicate differences in structure but rather differences in the magnitude and direction of tilt of the stereocilium relative to the plane of section. Moreover, the festooning should disappear when the section thickness reaches the axial repeat period of the crossbridges.

Bundles with hexagonally arranged filaments

In order to see how these predictions were made, we examined the nature of crossbridges in needles, another bundle of actin filaments formed *in vitro* from sea urchin egg extracts^{9,10}. In the needles, actin filaments are linked together by a second protein, fascin. The lattice that describes the positions of the filaments is

a hexagonal one. The pattern of crossbridges between filaments, however, cannot be truly hexagonal since the actin filaments to which they are attached do not have hexagonal symmetry. If actin had hexagonal symmetry, it would be possible to construct a crystalline bundle in which all crossbridges are in equivalent, hexagonally related positions. Because actin is helical, however, some of its subunits are in approximately the correct position to make such bonds, so that if 13° of bending is allowed, crossbridges can be formed to link every actin filament to its six nearest neighbours. The resulting structure is thus formed from nearly equivalent or quasi-equivalent bonds.

In the needle, the locations of crossbridges are easily found by finding the positions of pairs of bonding sites on neighbouring filaments in the array. Figure 2a shows three filaments taken from a hexagonal array. Let us begin by arranging filaments A and B so that a crossbridge is made at position 2. In the hexagonal array, the bonding rule requires that crossbridged filaments be in axial register and be separated by the appropriate distance (about $85 \text{ \AA}$). Using this bonding rule, we find we can also make crossbridges between filaments A and C at position 7 and between filaments B and C at position 11. Some bending of

crossbridges is needed to achieve bonding at the different sites, but the amount of bending required, which varies depending on position, is never more than about 13° . The bonding scheme is shown diagrammatically in Fig. 2b and from it we see that all neighbouring filaments are linked together. Note that although the diagram is for the first 14 subunits, the crossbridging occurs along the entire length of the bundle so that for any pair of actin filaments, there will be one crossbridge per $375 \text{ \AA}$ repeat.

How can such a bonding scheme lead to festooning in a bundle? Imagine we cut a thin nearly transverse section through a bundle as is shown diagrammatically for one row of filaments in Fig. 3a. The section is thin relative to the axial repeat of the crossbridges, and the plane of section is tilted relative to the bundle axis. At the left-hand edge the crossbridges contained in the section point towards 3 and 9 o'clock. As we progress in the section to the right, the crossbridges included in the section point

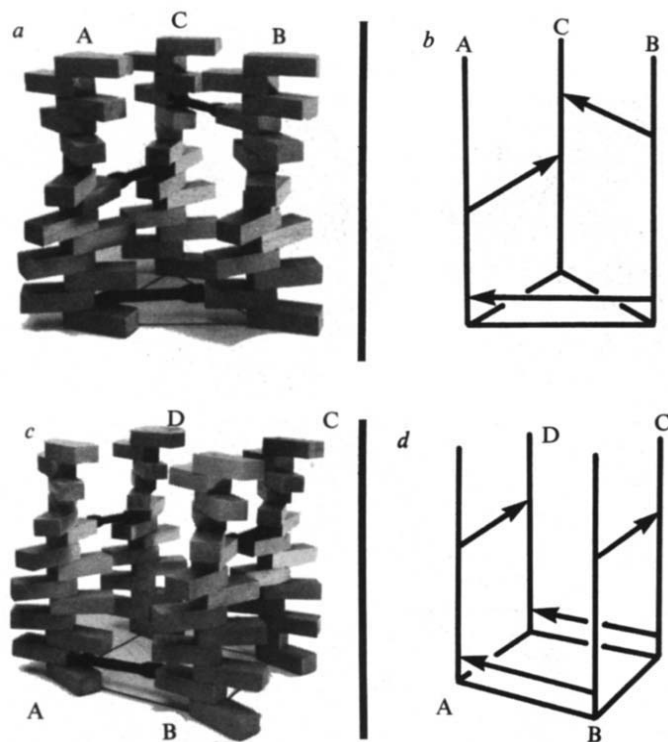


Fig. 2 a, Model depicting the arrangement of crossbridges in hexagonally arranged filaments. Three model filaments having the helical symmetry of actin are sitting on three vertices of a hexagonal lattice. The filaments are rotationally set so that the lowest subunit or block on each is pointing in the same direction. The bonding rule, arbitrarily chosen, allows a crossbridge (black arrow) to join the outer end of a subunit on one filament to an inner end on a neighbouring subunit. Such a crossbridge linking filaments A and B is seen at subunit 2, the next to lowest subunit. Because of the helical symmetry of the models, a crossbridge also links filaments C and A at subunit 7 and filaments C and B at subunit 11. The crossbridges which link these three filaments together will link together all the filaments in a hexagonal array. b, Schematic drawing of the crossbridging scheme depicted in a. c, Model depicting the arrangement of crossbridges in tetragonally arranged filaments. Four model filaments identical to those in a are sitting on the vertices of a tetragonal lattice. The filaments are rotationally set so that the lowest subunit in each is pointing in the same direction. The bonding rule, which is the same as that in a, allows a crossbridge at subunit 2 between filaments A and B and between filaments D and C. Filaments A and D and B and C are crossbridged at subunit 9. With this bonding rule, all the filaments in a tetragonal lattice can be crossbridged. The point of this figure is that the same bonding rule can crossbridge either a hexagonal or a tetragonal array of filaments. d, Schematic drawing of the crossbridging scheme depicted in c.

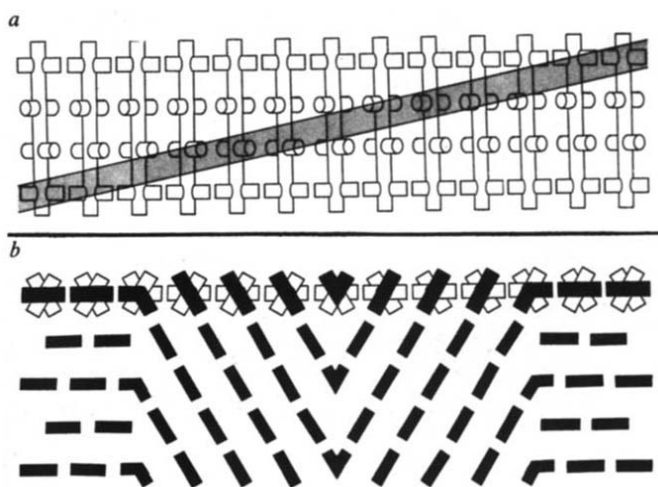


Fig. 3 Scheme showing how festooning arises. a, Thin section taken through a single row of filaments—side view. A row of filaments is shown schematically with the crossbridging material drawn in as a series of rods projecting from the cylinder. The portion of the row included in the thin section is indicated by the grey area. b, Top view of thin section. If the thin section is viewed from the top, the pattern indicated by blackened bars would be seen. In the upper row the crossbridges not included in the section are drawn in for reference but are not blackened. This row corresponds to the row of filaments in a. The remaining rows complete the pattern expected for a hexagonal array of filaments. Thus the second row from the top is shifted left half the interfibril spacing. Row three is a repeat of row one and so on. The result is a festooned pattern.

towards 5 and 11 o'clock. Continuing to the right, the included crossbridges again change direction to 7 and 1 o'clock and then, to complete one repeat of the pattern, to 3 and 9 o'clock. The pattern of crossbridges seen looking down on the section is shown in the top line of Fig. 3b. If we extend this pattern from one row to that expected for a hexagonal bundle, we get the festooned pattern shown in Fig. 3b. The festooning arises from a conversion of the axial periodicity of crossbridges into a transverse period by the tilt of the almost transverse section. We can make a number of predictions:

- (1) The pattern should disappear as the section thickness approaches the repeat of $375 \text{ \AA}$, since each section will contain crossbridges from one filament to all of its neighbours.
- (2) The direction of the festooning depends on the orientation of the knife relative to the axis of the bundle.
- (3) In a pair of serial sections of thickness, t , the festooned pattern should shift by $t/375$ of its period. Thus if we cut $125\text{-\AA}$ sections, the pattern should shift laterally by $1/3$ of a festoon in consecutive or serial sections. The period and direction of the motif remain unchanged.

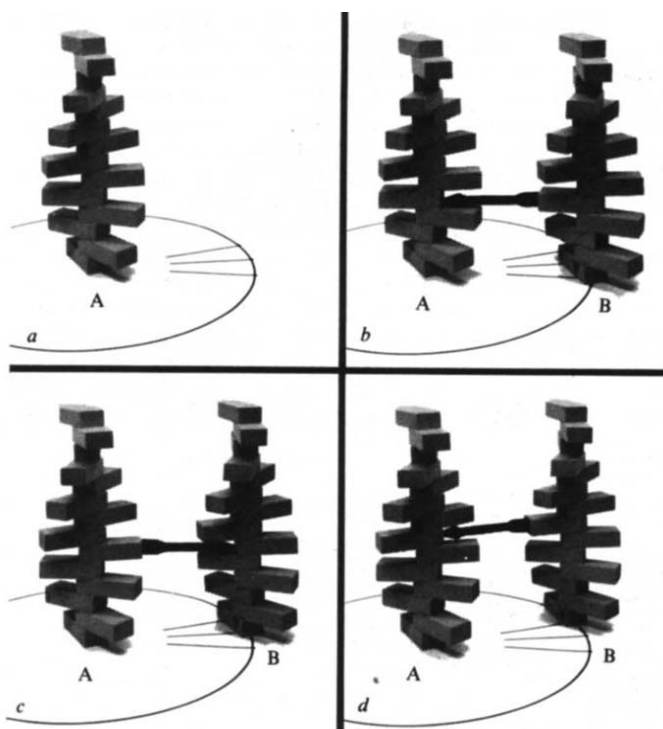


Fig. 4 Model showing the potential of helices to be crossbridged. *a*, Single model filament at the centre of a circle indicating the bonding distance. Three radial lines are drawn to indicate three positions separated by the equivalent of about 10 Å. *b*, Crossbridge formed between a pair of filaments. If a second (outer) filament is placed at the first position and rotationally set so it is in register with the central filament, then, given the bonding rule (see Fig. 2), a crossbridge can be formed at the fifth subunit from the bottom. *c*, Crossbridge formed when the outer filament is moved to second position. If the outer filament is moved around the central filament by the equivalent of 10 Å (or 13°), the most favourable position for a crossbridge is at the sixth subunit from the bottom. Note that the outer filament is moved so that the two filaments remain in register. *d*, Crossbridge expected when the outer filament is in the third position. In the third position, the most favourable subunit for crossbridging is the seventh one from the bottom. Note that as the outer filament moves around the central one in a counter-clockwise manner (as viewed from the top), the most favourable position for a crossbridge rises on the central filament. Continuing this process, we conclude that the outer filament can be crossbridged to the inner one at any position along the circle if a modest amount of crossbridge bending is allowed.

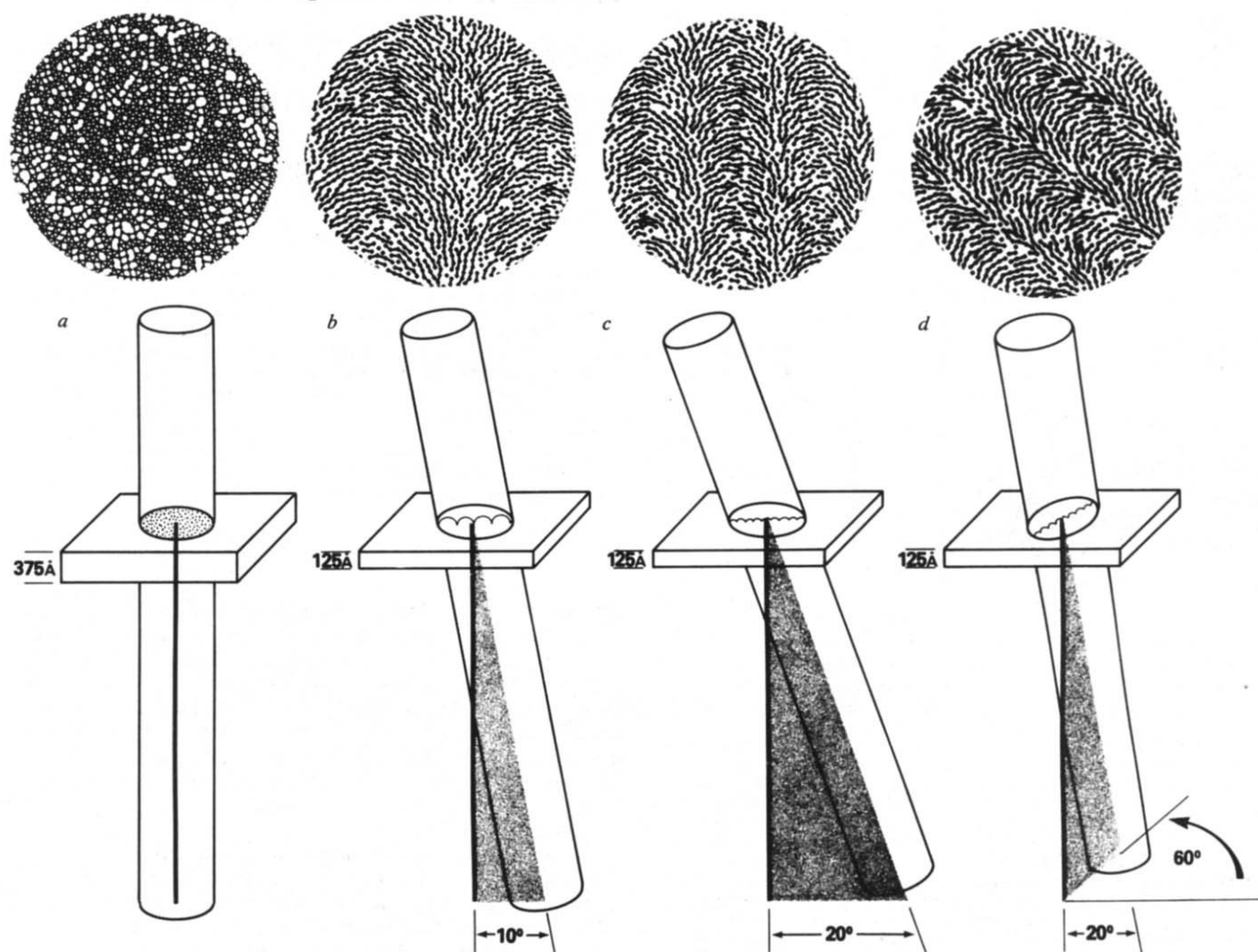


Fig. 5 *a*, Computer simulation of a thick section. If the section thickness is at least the crossbridge repeat of 375 Å, no festooning is seen since crossbridges pointing in all directions from each filament are included in the section. The computer has generated a liquid array of filaments and crossbridges and simulated a 375-Å thick section. *b*, Computer simulation of a thin section cut at a tilt of 10°. The same array of filaments is used as in *a*, but the computer simulated a 125-Å thick section cut at a tilt of 10°. The result is a festooned pattern having a long repeat. *c*, Computer simulation of a thin section cut at a tilt of 20°. Since the tilt is in the same direction the pattern runs in the same direction but has a finer period. *d*, Computer simulation of a thin section cut at 20° tilt but direction of tilt rotated 60°. The effect is to rotate the direction of the pattern 20° but leave the period unchanged.

- (4) The period of festooning, l , should depend on the tilt of the section relative to the bundle. More specifically, the period should be equal to the axial repeat, d , of the crossbridges divided by the sine of the angle by which the bundle is tilted off the normal to the plane of the section, that is, $l = d/\sin \theta$. For actin, $d \approx 375 \text{ \AA}$ and thus a festooning period of $1,000 \text{ \AA}$ corresponds to a tilt of $\sin^{-1} (375/1,000) \approx 22^\circ$.
- (5) The scalloping in the pattern in Fig. 3c has its convex side up. If the hand of the helical arrangement of crossbridges is reversed, the pattern would reverse to one with its convex side down. Thus, the asymmetry of the motif can be used to determine the hand of the crossbridges, which depends on the hand of the actin filaments.

In a previous paper⁶ we verified these predictions experimentally, but we were unable to understand how the predictions could be valid when the actin filament locations, as seen in transverse section, had no regular order. Although there is no such order in transverse sections, in longitudinal sections we found that actin filaments appeared paracrystalline; that is, they had their cross-over points in register. The question is therefore: how can a bundle of actin filaments maintain itself in perfect longitudinal register yet be disordered in transverse section?

Our key to resolving the paradox came from our study¹⁰ of the bonding rule for the crossbridging of filaments in the hexagonally packed bundles such as the needle from extracts of sea urchin oocytes, in which all filaments are in register. The axial register of the filaments is a requirement because if the filaments were rotated or translated relative to each other, none of the bonding sites, as shown in Fig. 2a, would be in the correct orientation and at the correct height to allow crossbridging. Thus, our ability to crossbridge filament A to B, B to C and C to A depends on the cross-over points being in register.

Bundles with tetragonally arranged filaments

What is special about this bonding rule is that we can also make a bundle in which the filaments lie on a tetragonal lattice instead of a hexagonal one. In Fig. 2c, the filaments A, B, C and D are placed at the corners of a square. They are arranged so that A is crossbridged to B and C to D at position 2. This is the same crossbridge that is formed at position 2 in the hexagonal bundles. In the tetragonal arrangement we find that at position 9 we can make a crossbridge from A to C and from B to D thus linking together the bundle. The amount of bending of crossbridges is similar to that required in the hexagonal case. Only two crossbridges, however, are made in the first 14 subunits instead of three as is the case in the hexagonal bundle. What we have shown is that the same specific bonds can generate either a tetragonal or a hexagonal bundle.

We can generalize from these two types of bundles by extracting the rules that govern the crossbridging between a pair of filaments. In Fig. 4 the two filaments are placed together so that the cross-over points are in register so that the filaments can be crossbridged. In Fig. 4b, a crossbridge can be made at position 5. This crossbridge can still be made if we move filament B about $10 \text{ \AA}$ along the circle drawn around filament A. At this point less crossbridge bending is required if we change the crossbridge from position 5 to position 6 (Fig. 4c). When we again move by $10 \text{ \AA}$, the crossbridge changes to position 7 (Fig. 4d). The bending of the crossbridges varies depending on the relationship between the two filaments and on the symmetry of the actin, that is the number of subunits per turn. For actin filaments having the symmetry of those in the stereocilium (2.159 units per turn), the crossbridges need be bent no more than 13° , which is the same value found in the fascin crossbridges in the needle. The important point is that two actin filaments can be crossbridged if they are in register and are separated by the length of a crossbridge; or, in other words, if we draw about a filament a circle whose radius is equal to the length of a crossbridge, a second filament put anywhere on the circle can be crossbridged to the first filament provided that the two filaments have their cross-over points in axial register.

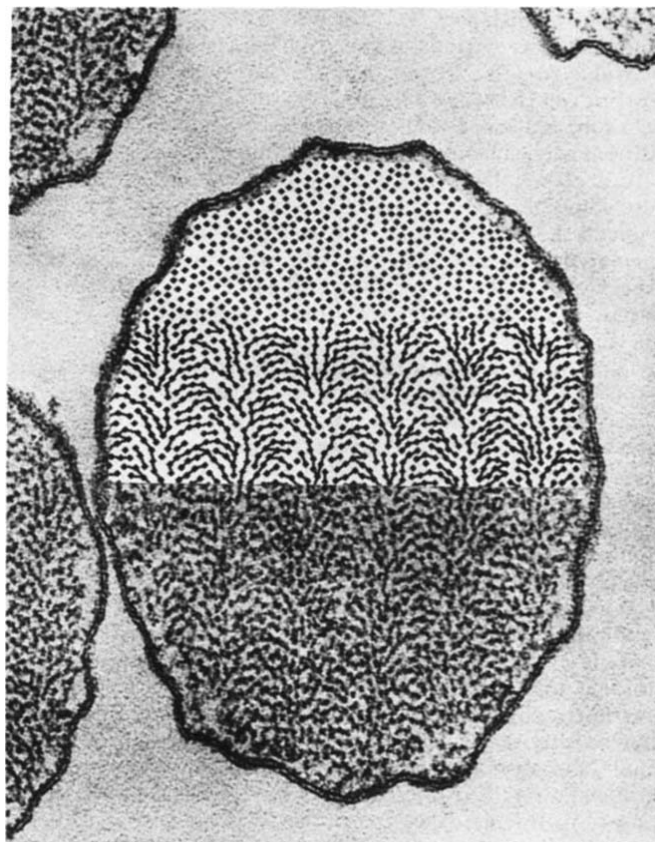


Fig. 6 Composite of the computer-generated array of filaments, filament plus crossbridges, and a thin section of a stereocilium. The top half of the stereocilium has been replaced by a computer-generated thin section that mimics the true pattern. The computer-generated section corresponds to a $150 \text{ \AA}$ section cut at an angle of 35° . The crossbridges in the upper part of the display are omitted so that the liquid arrangement of filament axes can be easily seen. $\times 155,000$.

Bundles with a liquid arrangement of filaments

With the bonding rule formulated in the preceding sentence, we can generate a liquid-like bundle with specific crossbridges. To demonstrate this, we generated a liquid-like arrangement of filaments using a Monte-Carlo random number generating method. For specifying filament locations, a 512 by 512 element grid, with a scale of $1 \text{ grid element} = 10 \text{ \AA}$, was used. The probability of locating a filament at a given grid position increased as a function of the number of other filaments to which it could be crossbridged. A radial probability function, $P(r)$, was created which incorporated volume exclusion (for $r < 70 \text{ \AA}$, $P(r) = 0$, so no two filaments could be closer than $70 \text{ \AA}$ apart). $P(r)$ also had a large peak at the crossbridging distance, $100 \text{ \AA}$, to reflect the likelihood for two neighbouring filaments to be crossbridged to each other. The grid was scanned, element by element, with the probability of creating a filament at a given grid element equal to P_T , where

$$P_T = 1 - \exp \left[-\sum_i P(r_i) \right]$$

$P(r_i)$ is the radial probability function evaluated for all filaments,

i , located at distances, r_i , from the given grid element. The summation was carried out over all filaments within a radius of 15 grid elements. In the resulting array all filaments were crossbridged to at least one other filament, but, due to volume exclusion, not more than seven. Figures 5a and 6 show the positions of the filaments generated by this technique. Note that there is no regularity in the array. The spatial distribution of these computer-simulated filaments is strikingly similar to that observed in the stereocilia. This was true both with regard to filament density and isotropy, and was confirmed by visual inspection as well as comparison of the Fourier transform of the computer-generated array with that of thick sections of stereocilia (Fig. 1a).

Figure 5a shows the array with all the crossbridges as would be seen in a thick section. These crossbridges are easily drawn in as, according to the scheme illustrated in Fig. 4, all filaments separated by the appropriate distance can be crossbridged. Note how extensively crossbridged the array is. Let us now examine thin sections of the array. The model tells us how the height of a particular crossbridge in the array depends on the direction it points. We set the array so that all crossbridges pointing from 3 to 9 o'clock lie at a height, $h = 0$. If we took a thin section perpendicular to the bundle at $h = 0$, we would find that all the crossbridges which point from 3 to 9 o'clock would lie in this plane. If we look at the next section above the first, we would find that the crossbridges in that section would point in a direction rotated counterclockwise to that in the section at $h = 0$. Thus, in thin sections that are perfectly perpendicular to the bundle, the festooning would not be seen but rather we would see filaments joined into parallel line segments by the crossbridges. If, on the other hand, the sections were cut off the perpendicular, the crossbridge direction would change continuously as the section passes through different heights within the bundle. As we start the section at $h = 0$, the crossbridges would point from 3 to 9 o'clock. As the section moves up, the direction of crossbridges included in the sections shifts in a counterclockwise direction which produces the curved line segments seen in the festooned motif. This effect shows up beautifully in the computer displays. In these displays we varied the angle and direction of tilt of the bundle relative to the plane of the section. Figure 5b shows the equivalent of a 125-Å section taken at a tilt of 10°, and Fig. 5c shows the same section for a tilt of 20°. Note the change in the period but not direction of festooning. In Fig. 5d the direction of tilt is altered by 60° but the tilt is kept at 20°. Note that the direction of festooning, but not its period, changes.

With the computer-generated model we are thus able to produce the festooning seen in electron micrographs of thin sections of the stereocilium (Fig. 6). This model has the following features which mimic the behaviour of the stereocilium on sectioning.

- (1) Sections much less than 375 Å in thickness show festooning whereas those about 375 Å lose their festooned motif.
- (2) In serial sections the festooning appears to shift laterally by an amount which is proportional to the section thickness.
- (3) A change in tilt produces a change in the period of festooning.
- (4) In longitudinal sections the filaments are in perfect register.
- (5) In Fig. 5b the festooned motif has its convex side upward. If the hand of the actin and the crossbridges were reversed, the convex side would be down. Actual tilting of the stereocilia during sectioning, as shown in Fig. 5b, shows exactly the

convex side-up festooning we predicted. This confirms the hand of the arrangement of crossbridges shown in Fig. 4 (that is, as the filament changes position from that in Fig. 4b to Fig. 4d the crossbridge moves up rather than down the filament).

Thus, the paradox of how one can get crystalline axial order and liquid transverse order is resolved. A liquid-like bundle of filaments with helical symmetry can be crossbridged together with specific bonds, provided that all the filaments are in register. Turning the statement around, we propose that a single crossbridge between any two filaments will force them into register. As this constraint applies to each successive filament in the bundle regardless of their lateral order, the self-assembled bundle must have all its filaments in register. Thus, no external structure is needed to align the filaments; they do it themselves.

What are the biological advantages of a liquid arrangement? Presumably, such a bundle can be put together more quickly than the corresponding hexagonal bundle as the restrictions on placement of filaments in the former are much less than in the latter. For example, the formation of cell extensions such as the actin-containing filopodium¹¹ can take place much more rapidly because the actin filaments need not make and break connections to become hexagonally packed, a process which takes considerable time. In needle formation, at early times the needles lack transverse order much as the stereocilia of the lizard. On incubation the ordering increases until, after many hours, hexagonally packed filaments are observed¹².

In the hexagonal bundle, the number of crossbridges is a maximum. With increasing disorder in filament position, there will be decreasing numbers of crossbridges. In the limiting case of a very disordered bundle, the paucity of crossbridges will lead to reduced rigidity. This may provide a mechanism for building of bundles of different rigidity or for altering their rigidity after assembly. Exposure to prolonged intense sound produces limp stereocilia, and perhaps this results from alterations in the crossbridges¹³.

We have shown that the concept of specific bonding applies to the actin bundles with liquid lateral order in which all crossbridges are in almost identical environments. The potential for such organization is a consequence of the helical symmetry of the actin filaments in which the radial orientation of subunits allows bonding to take place at any angular position around a filament. What is particularly interesting is that the bonding rule for a pair of filaments, at least as we have outlined it here, does not determine the lateral organization. The resulting bundle may be hexagonal as in stereocilia of the chicken (data not shown). Alternatively, using the same bonding rule, the bundle may have only liquid lateral order as in the stereocilia of the alligator lizard. The regularity of filament arrangement would thus not appear to be a consequence of the bonding properties of the actin and its crossbridges. Rather, we suppose that control over this aspect of organization is determined by the conditions at the time of assembly.

Finally, our conclusions about crossbridging between actin filaments can apply equally well to the crossbridging of other helical filaments, for example microtubules or intermediate filaments.

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Resolution, reconstitution and kinetics of the primary action of a hormone receptor

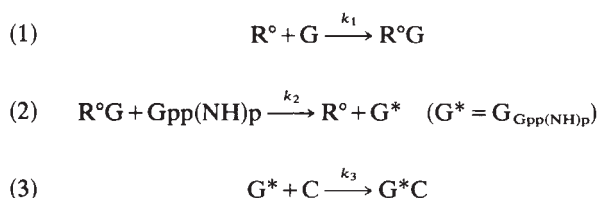
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The β -adrenergic receptor (R) and the GTP binding protein (G) with which the receptor interacts were obtained in separate soluble fractions. Their recombination produced a hormone responsive system. In the presence of hormone the encounter of R with G in the reconstituted membrane occurs much faster than the subsequent R-induced activation of G.

THE interaction of a hormone or neurotransmitter receptor with the next component along the pathway of signal transmission can be defined as the primary action of the receptor. Resolution of its participating components and subsequent reconstitution are essential to the understanding of the molecular mechanism of receptor function. Unfortunately, this initial step has not been sufficiently characterized even for the much studied insulin¹, acetylcholine² and α -adrenergic receptors³.

There is, however, considerable information about the activation sequence of the β -adrenergic receptor^{4,5}: the hormone-receptor complex (R^o)^{6,7} activates a guanyl nucleotide binding component (G) by introducing GTP into it^{8,9}. The activated G (G^*) interacts with the catalytic unit (C) of the adenylate cyclase to form the active enzyme (G^*C)¹⁰. Hydrolysis of the bound GTP terminates activation¹¹. Replacement of GTP by the hydrolysis-resistant analogue Gpp(NH)p produces a persistently active enzyme complex (G^*C)¹². The process may be described by the following steps:



Step (1) is the encounter of R^o and G in the membrane; step (2) is the R^o -induced introduction of Gpp(NH)p into G; and step (3) is the interaction of G^* with C to form the persistently active adenylate cyclase. k_1 , k_2 and k_3 are the forward rate constants of steps (1)–(3) respectively.

The function of R has so far been investigated only by measuring adenylate cyclase activity in the intact cell membrane. On the basis of such kinetic studies, many models for the action of R in the activation of adenylate cyclase were proposed^{4,13–15}. It is, however, extremely difficult to reach definitive conclusions from such studies because the relative amounts of R, G and C in the membrane are fixed and because the function of R is not measured directly in its primary action on G (Steps (1) and (2)), but rather, indirectly, through the action of G^* on C (Step (3)).

We have now succeeded in obtaining separate solubilized preparations of R and of G, both free of functional C. Various amounts of the two preparations can be combined to reconstitute functional systems with the desired R to G ratios. When hormone and Gpp(NH)p are added, persistently activated G^* is produced. The amount of G^* is subsequently measured in a separate system, by its implantation in the membrane of cells which contain an excess of C but no functional G^{16–18}. The kinetics of R action on G are found to be first order with respect to R concentration, and zero order with respect to G concentration. The nature of receptor function is discussed in the light of these findings.

Resolution and recoupling of R and G

Solubilization of a functional receptor and its implantation in a cell membrane have been recently reported from our laboratory¹⁹. Since the solubilized fraction had both R and G activities, we searched for a preparatory step to obtain a membrane preparation which would contain R but no G or C. It was found that treatment with strong alkaline buffer (pH 11.9) eliminated essentially all G and C activity of the membrane, leaving R undamaged. Subsequent treatment of the alkali extracted membrane with deoxycholate produced solubilized R free of G and C. The solubilized G preparation, essentially free of R and C, was obtained by cholate extraction²⁰. Mixing of the soluble R preparation with the soluble G preparation, followed by removal of detergents and phospholipid treatment, gave a functional hormone responsive system. The entire procedure of solubilization and reconstitution is shown schematically in Fig. 1. The primary action of the β -adrenergic receptor is studied in the reconstituted system by measuring its product G^* . The amount of G^* is determined by the proportional enhancement of adenylate cyclase activity in cyc^- cells which lack functional G. This activity will be referred to as 'G activated' (Figs 2–5).

Table 1 Specific activities of R in the reconstituted system and in erythrocyte membrane preparations

Systems and treatments	Amount of R* (a)	Rate of G activation† (b)	Specific activity of R‡ (b/a)
I Reconstituted RG, activated by hormone + Gpp(NH)p, fused with cyc^-	85	12	0.14
II NEM treated membranes activated by hormone + Gpp(NH)p and fused with cyc^-	169	56	0.33
III Membranes activated by hormone + Gpp(NH)p	72	23	0.32
IV Membranes activated by hormone + Gpp(NH)p fused with each other	72	12	0.17

Systems were activated (2 or 3 min) with 10 μ M isoprenaline + 10 μ M Gpp(NH)p at 30 °C. Activation was terminated with 10 μ M propranolol and after 0.5 min, 10 μ M GTP was added. The systems were immediately placed in ice. Adenylate cyclase was measured either directly or after fusion with cyc^- cells ('Rate of G activation'). The amount of receptor (R) was determined by binding of [¹²⁵I]-hydroxybenzylpindolol³⁴. System I: reconstitution was produced by R and G mixing 1:1 (v/v) (as described in Fig. 1). System II: membranes were treated with N-ethylmaleimide selectively to inactivate the catalytic unit (C)^{18,19}. After activation, as described above, membranes were treated with phospholipids (40 μ l ml⁻¹ containing 1 mg protein), pelleted and fused with cyc^- cells (Fig. 1). System III: membranes were activated as above, washed once and assayed for adenylate cyclase activity. System IV: membranes activated as in III went through the fusion process, without cyc^- cells, and were then assayed for adenylate cyclase.

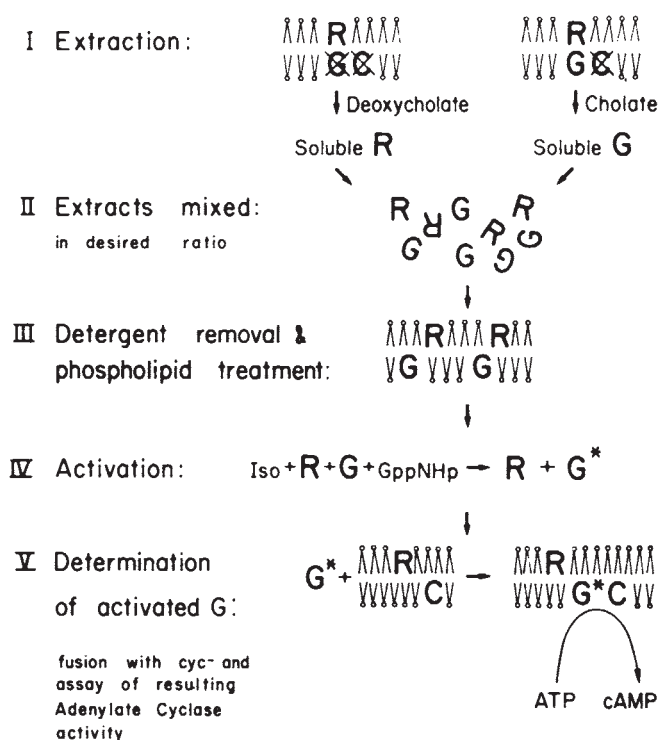
* fmol per system.

† Expressed as G activated per min per system as pmol cyclic AMP min⁻¹.

‡ Expressed as G activated per min per fmol R.

Fig. 1 Scheme describing the differential solubilization and reconstitution of the β -adrenergic receptor and the guanyl nucleotide binding protein. R is the β -adrenergic receptor; G is the guanyl nucleotide binding unit; G* is the Gpp(NH)p activated G unit, and C is the adenylate cyclase catalytic unit.

Receptor extraction: Turkey erythrocyte membranes¹⁹ were washed with 10 mM MOPS buffer, pH 7.5, containing 1 mM mercaptoethanol, and were suspended at 1 mg ml⁻¹ in 50 mM phosphate buffer, pH 11.9, containing 2 mM EDTA. The suspension was kept for 20 min at 4°C, centrifuged at 27,000 g for 15 min, and resuspended in 20 mM MOPS buffer, 1 mM mercaptoethanol, in half the previous volume. The G unit is lost during this treatment together with 50% of the membrane protein. The alkali extracted membranes, when fused with cyc⁻ cells, contribute only negligible amounts of G as measured by adenylate cyclase activity. The alkali extracted membranes were treated with phospholipid (10 mg ml⁻¹ soybean phospholipid in 10 mM Tris HCl buffer, pH 7.5, 1 mM EDTA, sonicated twice for 5 min under N₂), as previously described¹⁹, only that 0.2 ml phospholipid was added per ml of membrane suspension. After incubation at 4°C in presence of 10 mM MgCl₂, 20 μ M isoprenaline was added, and the membranes were incubated for 15 min at 30°C with occasional stirring. The suspension was cooled at 4°C, centrifuged at 18,000 g for 10 min, and resuspended in solubilization buffer¹⁹ at one-ninth the original volume (which served for alkaline extraction). The membranes were solubilized by the addition of one volume of sodium deoxycholate, 12 mg ml⁻¹, in solubilization buffer¹⁹. The supernatant, after centrifugation at 200,000 g for 30 min, served as the soluble R preparation (0.4 mg protein per ml). **G unit extraction:** The procedure was adapted from that of Sternweis and Gilman²⁰. Turkey erythrocyte membranes were washed and resuspended at 10 mg protein per ml in cholate solubilization buffer (20 mM HEPES, pH 8.0, 2 mM mercaptoethanol, 1 mM MgCl₂, 0.2 mM EGTA, 20 μ M phenylmethylsulphonyl fluoride). One volume of 20 mg ml⁻¹ cholate in the above buffer was added, the suspension was shaken for 30 min at 4°C, and centrifuged at 200,000 g for 30 min. The supernatant served as the G preparation (0.9 mg protein per ml). The procedure apparently inactivated the catalytic unit. **Mixing of solubilized R with solubilized G:** To produce the desired ratio of R:G, the appropriate volumes of the two extracts were mixed. Controls containing only G or only R were mixed with the complementary pure detergent solution instead of the complementary extract. **Detergent removal:** Polystyrene SM-2 beads were added as previously described¹⁹. The preparations were subsequently treated with phospholipids, 150 μ l ml⁻¹, as described above. **Activation:** The phospholipid-treated preparations were incubated at 30°C with the synthetic hormone isoprenaline (10 μ M) or the receptor blocker propranolol (10 μ M) plus the non-hydrolysable GTP analogue guanosine 5'-(β , γ -imino)-triphosphate (Gpp(NH)p) (10 μ M). After the desired time period, activation was terminated by addition of 10 μ M propranolol and 10–100 μ M GTP. The mixture was kept at 4°C for 20 min. After centrifugation at 18,000 g for 10 min, the pellets were frozen in liquid N₂ and stored at -70°C. These pellets had no detectable adenylate cyclase activity. **Determination of activated G:** Each pellet described above was fused^{19,32} with 3 $\times$ 10⁷ cells of the mutant cyc⁻ S49 lymphoma which lacks functional G (refs 16, 17). The resulting increase in basal adenylate cyclase activity was measured in presence of 2 μ M propranolol. This activity, referred to as 'G activated', can be attributed solely to the amount of activated G which was transferred by fusion to cyc⁻ membranes. R of the cyc⁻ cells (section V above) has no function in this assay. The resulting basal adenylate cyclase activity was directly proportional to the amount of activated G placed in the fusion system. The total amount of G (non-activated plus activated) implanted in the cyc⁻ membranes was determined by measuring adenylate cyclase activity in presence of 0.1 mM isoprenaline plus 0.1 mM Gpp(NH)p. Adenylate cyclase activity in presence of 10 mM NaF also gave a measure of G units transferred by fusion. These assays were performed at 37°C for 30 min³³. **Yield of R and of G:** The β -adrenergic receptor in the R preparation was 0.3 pmol per ml of extract, as determined by binding of [¹²⁵I]-hydroxybenzylpindolol³⁴ after detergent removal. The G preparation is estimated to contain 2.5 pmol GTP binding sites per ml. The calculation is based on a 50% yield in the cholate extraction, as determined by fusing the extracted turkey erythrocyte membranes with cyc⁻ cells and assaying adenylate cyclase activity (F⁻). The original content of G in the native membrane is taken to be 1 pmol of hormone induced ³H-Gpp(NH)p (ref. 35) or ³H-GTP binding sites per mg of protein.



Hormone activation of the reconstituted R-G system is shown in Fig. 2. Dramatic hormone-induced activation of G is exhibited only by the system which was produced by combining the R and G preparations. The individual preparations of R and of G show only a slight hormonal response. Fluoride activities serve as an independent measure for the amount of G fused into the cyc⁻ membranes. The R preparation produces no detectable fluoride activity and is therefore considered essentially free of G. The G preparation produces fluoride activity as high as the R plus G system, indicating a similar efficiency for both systems in the transfer of G units to the cyc⁻ cell membrane by the fusion process. Hence, the only explanation for the very low hormone-induced G activity in the separate preparations of R and of G is the paucity of the G component in the R preparation and the R component in the G preparation.

Efficiency of R-G recoupling

The amount of G activated (G*) per minute by a unit amount of R may be used as a measure of the productive interaction of R with G (specific activity of R). As shown in Table 1, the specific activity of R in the reconstituted system is almost half that of the native turkey erythrocyte membrane (compare systems I and II in Table 1). It is also shown that the specific activity of R measured in the erythrocyte membrane, through its native catalytic unit, is comparable with that measured through the catalytic unit of the cyc⁻ cells (Table 1, systems II and III). However, this observation should be treated cautiously because the experiments involve the unknown parameters of fusion

efficiency with cyc⁻ cells and the turnover numbers of the catalytic units of cyc⁻ and turkey erythrocyte membranes. Note also that absolute activities fluctuated considerably in different experiments throughout the present study. The major variables responsible seem to be detergent effects during solubilization and reconstitution, and competence of different batches of cyc⁻ cells. Therefore, absolute activities are comparable only for different systems within the same experiment.

Kinetics of R-G activation

The availability of separate preparations of R and G presented a unique opportunity to study the kinetics of their interaction as a function of their concentration in the reconstituted membrane.

Figure 3 shows the progress of G activation over an extended time period. It is apparent that the systems containing the larger amount of R exhibit a higher rate. In contrast, the concentration of G does not seem to affect the rate of its activation by R. The cause for the two phases in the kinetics of activation is not yet apparent.

To test the kinetics of the R-G interaction more rigorously, systems with a constant amount of R and different amounts of G were reconstituted. Initial rates of G activation were determined by limiting the activation time to 5 min (see Fig. 1, step IV). Figure 4 clearly demonstrates that the concentration of G had no effect on the initial rate of its activation. The kinetics are zero order with respect to G concentration. The apparent lower initial rate in the two systems containing the lesser amounts of G

is due to activation of all the G units within the 5 min incubation period. This is evident from the addition of isoprenaline plus Gpp(NH)p to the final adenylate cyclase assay which does not result in additional activation (Fig. 4, open circles, second activation). The addition of isoprenaline plus Gpp(NH)p in the adenylate cyclase assay also demonstrates that the amounts of G transferred to cyc^- cells are directly proportional to the amount in the reconstituted system (Fig. 4, second activation).

Figure 5 shows the effect of R concentration on the initial rate of G activation. The rate is directly proportional to the amount of R and is thus first order with respect to R. Limitations of the system did not permit the R/G ratio to increase to the point of saturation, where the initial rate of G activation might reach a maximum. It is interesting that a similar dependence on R concentration was observed in intact turkey erythrocyte membranes after graded inactivation of R by an affinity label¹⁵.

Analysis of kinetics

In the process of hormone activation of adenylate cyclase R^o must meet G, and G* must associate with C (see steps (1)–(3)). If the association of R^o with G is rate limiting (step (1)), then the rate of G activation should be dependent on the concentrations of both R and G. On the other hand, if the activation of G by R (step (2)) is rate limiting, then the concentration of RG complex would determine the rate. In the latter case, step (1) must occur much faster than step (2) and, therefore, all R will be in the RG form (when $[G] > [R]$). Thus, first order kinetics with respect to R concentration and zero order with respect to G concentration are predicted. As shown by the present findings (Figs 4, 5), the latter possibility holds true; step (2) is rate limiting. We therefore conclude that in the reconstituted R–G system, the rate of G activation by R is determined by the step of Gpp(NH)p introduction into the RG complex rather than by the rate of encounter of R with G.

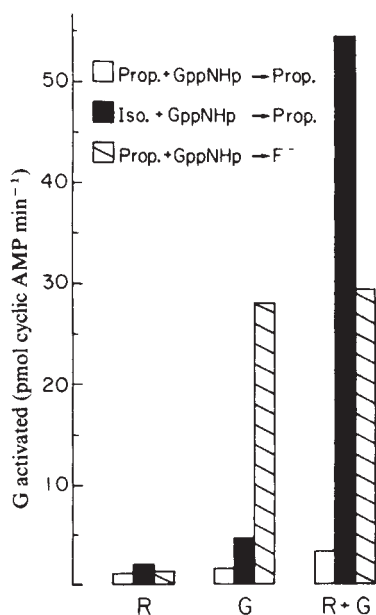


Fig. 2 Hormone-induced activation of G in the resolved and in the reconstituted preparations. Systems were reconstituted as described in Fig. 1 legend. At the mixing stage (step II, Fig. 1) the soluble G extract was combined (1:1 v/v) with deoxycholate, 6 mg ml⁻¹ (marked as system G), or with the soluble R (marked as system R+G). The system marked R was prepared by mixing the soluble R extract (1:1 v/v) with 10 mg ml⁻¹ cholate. All the systems were then treated as described in Fig. 1 legend, and 0.5-ml portions of each were activated (step IV, Fig. 1) with isoprenaline (black bars) or propranolol (open and crossed bars) for 5 min at 30°C before addition of Gpp(NH)p for 30 min. Activation was terminated by addition of 10 μ M propranolol. After 5 min, 1 ml of ice cold buffer (20 mM MOPS, pH 7.5, 1 mM mercaptoethanol, 10 μ M GTP, 10 μ M propranolol) was added to all systems. The pellets produced by centrifugation were fused with cyc^- cells. Adenylate cyclase was determined in presence of propranolol (open and closed bars) or NaF (crossed bars).

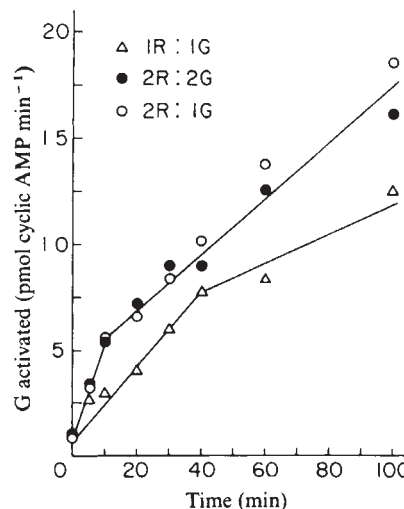


Fig. 3 Kinetics of G activation by the β -adrenergic receptor in reconstituted systems. Preparations (step II, Fig. 1) were mixed in the following ratios: R:G, 1:1 v/v (●); R:G:cholate solution, 2:1:1 v/v/v (○); and R:G:cholate solution:deoxycholate solution, 1:1:1:1 v/v/v/v (Δ). The systems were treated as described in Fig. 1 legend and activated (step IV, Fig. 1) with isoprenaline plus Gpp(NH)p. At various times 0.5-ml aliquots were taken and added to 1 ml of ice cold buffer (20 mM MOPS, pH 7.5, 1 mM mercaptoethanol, 0.1 mM GTP, 10 μ M propranolol). The amount of G activated in the samples was determined in presence of propranolol as described in legends to Figs 1 and 2. The relatively low activities in this experiment are due to a low yield of the G preparation used for the reconstitution. This is shown by measurement of total G (determined in presence of Gpp(NH)p plus isoprenaline in the adenylate cyclase assay). Total G activities were (average of all the samples): 10.2 pmol min⁻¹ (1R:1G), 20.4 pmol min⁻¹ (2R:2G), and 13.2 pmol min⁻¹ (2R:1G).

Extrapolating from these experiments with the reconstituted system to the native membrane, either step (2) or (3) could determine the rate of the overall process. An estimate of the apparent rate constant k_2 for step (2) (see legend to Fig. 4) gave a value that was in the same range as the rate constant values calculated for the overall activation process in the native membrane^{11,15}. It is therefore possible that step (2) is rate limiting in the overall process of hormonal activation of adenylate cyclase. However, further experiments will be needed to verify this suggestion.

The mobile receptor hypothesis²¹ was applied to the β -adrenergic receptor system¹⁵ and it was suggested that the rate of encounter between the receptor and the adenylate cyclase might be the rate limiting step of the activation process. The hypothesis²¹ predicted the independent existence of the hormone receptor⁶. However, the RG complex and the rate of G activation were not considered at the time. As these findings indicate that the R–G encounter is not rate limiting in the reconstituted system, a re-evaluation of previous models seems worthwhile.

Experiments on the overall process of adenylate cyclase activation in the native membrane indicated that a small amount of R could activate a larger amount of enzyme^{15,22}. The present work shows that R acts catalytically in activating at least 10 times its own amount of G (Figs 2–5). The activation of G by R proceeds in the absence of functional C (ref. 18, legend to Fig. 1). Thus, the catalytic function of the β -adrenergic receptor in the overall process of adenylate cyclase activation seems to be a mere reflection of its primary action on G.

Conclusions

The present study describes the reconstitution of the R–G interaction using solubilized, resolved preparations of R and of G. Kinetic properties of the R–G interaction were revealed which could not be studied in the native membrane where the concentrations of R and G cannot be manipulated and where the concurrent interaction of G with C compounds the analysis. The experiments imply that in the presence of hormone, all R is in

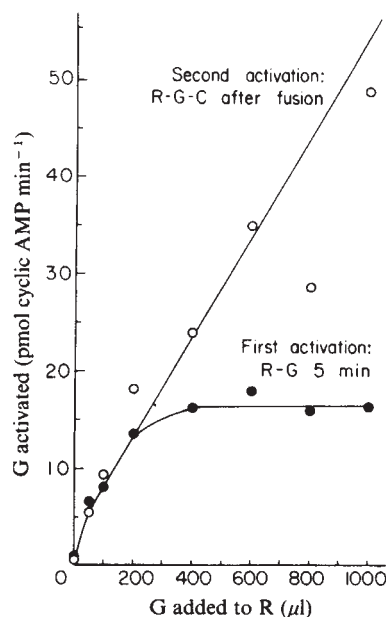


Fig. 4 Initial rates of G activation as a function of G concentration in the reconstituted system. R (0.5 ml) was mixed with different amounts of G (abscissa). In each system the volume of G added was made up to 1.0 ml by addition of 10 mg ml⁻¹ cholate. After reconstitution, as described in Fig. 1 legend, 1.0-ml aliquots were activated for 5 min (as in Fig. 3) and fused with cyc⁻ cells. Systems were assayed with propranolol to show the amount of G which had been activated (first activation) (●). In addition, aliquots from each system were subjected to a second activation with 0.1 mM isoprenaline plus 0.1 mM Gpp(NH)p in the adenylate cyclase assay (○). k_2 : The rate constant of step (2) in the text was calculated from the initial rate of G activation shown above, and the yield of G and R in the respective extracts (Fig. 1). R is all in the RG form (see text); therefore $k_2 = 1.4 \text{ min}^{-1}$.

the RG complex form. This conclusion is supported by recent findings that the hormone induces co-elution of R and G from a gel filtration column²³. As the R-G interaction has not been studied to date with purified components, the possibility cannot be excluded that unknown additional factors might be involved. It should also be noted that differences between the β -adrenergic receptor of turkey erythrocytes and that of other tissues have been reported^{14,24}. However, recent studies suggest that these differences are not fundamental^{25,26}.

The resolved R and G as well as the reconstituted R-G preparation seem to have considerable potential for future studies on hormone receptor action at the molecular level. A few important questions might be tackled. Are the differences in thermodynamic parameters between the binding of hormone and of blocker²⁷ due to the hormone-induced primary action of R with G? Does the reconstituted R-G system possess the hormone-induced GTPase activity²⁸ or is C required for this reaction? What are the specific phospholipid requirements for R and G functions?

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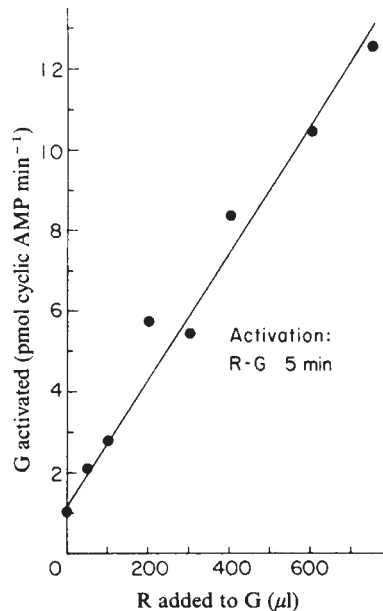


Fig. 5 Initial rates of G activation as a function of receptor concentration in the reconstituted system. A constant amount of G, 750 μ l, and increasing amounts of R (abscissa) were mixed and reconstituted. The volume of R was made up to 750 μ l with 6 mg ml⁻¹ deoxycholate. All other manipulations were as described in Fig. 4 legend.

The interaction of R with G takes place within a two-dimensional phospholipid bilayer which serves as the solvent. Manipulation of this solvent such as treatment with *cis*-vaccenic acid^{29,30} greatly influences the kinetics of adenylate cyclase activation. It is possible, therefore, that more extensive dilution of R and G by phospholipid addition or other manipulations might affect the properties of the system. Unfortunately, there is little information about kinetics of interaction of protein molecules in biological membranes. Rogers and Strittmatter³¹ published elegant studies on the interaction of NADH cytochrome *b*₅ reductase with cytochrome *b*₅ in native and reconstituted membranes. However, this system may not be a close analogy to the hormone-activated adenylate cyclase. The cytochrome and the enzyme reducing it are anchored in the membrane but the active groups interact in the water phase. In contrast, the β -adrenergic receptor transmits a transmembrane signal and, apparently, interacts with the G unit within, or on, the inner side of the membrane. It is hoped that the approach used in this work will be applicable to the study of other receptor response mechanisms and that the reconstituted R-G system can serve as a paradigm for protein-protein interactions in biological membranes.

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Cloning and DNA sequence of double-stranded copies of haemagglutinin genes from H2 and H3 strains elucidates antigenic shift and drift in human influenza virus

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Double-stranded DNA copies of the RNA gene coding for the haemagglutinin glycoproteins from human H2 and H3 pandemic strains of influenza virus have been cloned. DNA sequence analysis provides the first reported complete nucleotide sequence of an H2 haemagglutinin gene and a partial sequence (45%) of the H3 gene. The H2 haemagglutinin gene consists of 1,773 nucleotides containing an uninterrupted coding sequence of 1,686 nucleotides specifying a protein of 562 amino acids. Comparison of the amino acid sequences of these haemagglutinins with those of other H3 and avian strains reveals the extent of sequence changes in antigenic shifts and drifts.

THREE main properties of influenza virus haemagglutinins have prompted analyses of their structure. These glycoproteins are responsible for binding virus particles to cells during infection¹ and possibly mediate virus-cell membrane fusion²⁻⁴; antibodies which react with the haemagglutinin neutralize virus infectivity⁵ and, as a consequence, viruses which have the potential to cause new epidemics or pandemics of disease in an immune population have antigenically novel haemagglutinins; and finally, as the major glycoprotein component of virus membranes they share features of biosynthesis, processing and structure with integral membrane proteins of eukaryotic cells and thus serve as models for studying these proteins. Because of these properties, we and others⁶⁻¹¹ have been determining the primary structures of several haemagglutinins. To complete this determination we have synthesized and cloned in a prokaryotic vector complementary DNA copies of haemagglutinin virion RNA. Haemagglutinins used were from strains A/Japan/305/57 (H2 subtype) and X-31 (H3 subtype). We report here the complete nucleotide sequence of the Japan haemagglutinin gene and a partial sequence (45%) of the X-31 gene. Comparison of the derived amino acid sequences with those from other members of the H3 subtype A/NT/60/68/29C¹⁰ and A/Victoria/3/75⁹ and with a haemagglutinin from the avian H5N1 strain A/FPV/Rostock⁸ allows correlation of structure and function for the haemagglutinin molecule.

Cloning and DNA sequence of haemagglutinin genes

The influenza haemagglutinins chosen for analysis were from the high-yielding recombinant virus strains A/Japan/305/57; A/Bel/42; A/PR/8/34 (H2N1)¹² and X-31 (H3N2)¹³. These strains contain haemagglutinin genes from the originally isolated strains A/Japan/305/57 (H2) and A/Aichi/2/68 (H3). Total virion RNAs (vRNAs) from the two influenza recombinants were purified and polyadenylated at their 3' termini using ATP:RNA adenylyltransferase¹⁴. An average of about 50 adenylate residues were added to each RNA molecule. The polyadenylated vRNAs were then used as templates for reverse transcription of single-stranded (ss) DNA copies using oligo(dT)₁₂₋₁₈ as primer¹⁵. The products were separated in denaturing conditions on agarose gels into eight discrete bands whose sizes indicated that they were essentially full-length

copies of the genomic RNA segments¹⁶ (Fig. 1a). In a number of experiments approximately 3-5 µg of cDNA was synthesized using 10 µg of template RNA.

At this point different strategies were chosen for cloning X-31 and Japan nucleic acid sequences. Cloning of X-31 sequences involved direct insertion¹⁷ of poly (dA)-tailed vRNA/cDNA hybrids¹⁸ into the *Pst*I site of plasmid vector pBR322¹⁹ after poly(dT) tailing¹⁸ of the linearized plasmid. However, no full-length cloned copies of the HA gene were obtained. Subsequent experiments with the Japan strain involved the production of double-stranded (ds) DNA copies before insertion into the *Pst*I site of plasmid pAT153 (a derivative of pBR322 with a 622 base pair deletion)²⁰.

Single-stranded DNA copies of Japan vRNAs were converted to the double-stranded form²¹ utilizing the ability of the cDNA to form 3'-terminal hairpin loops²² to prime DNA polymerase I. Subsequent cleavage of the hairpin loops yields non-covalently joined ds cDNAs. Figure 1b shows the resolution on denaturing agarose gels of the ds cDNAs before and after S₁ nuclease treatment. The hairpin-linked ds cDNAs migrated to positions corresponding to approximately twice the molecular weight of ss cDNAs. After S₁ nuclease treatment, most of the ds cDNAs were converted to species which co-migrated with full-length first-strand cDNAs. The ds cDNAs were separated on a sucrose gradient and fractions containing full-length species were selected and tailed with poly(dC) using terminal transferase¹⁸. The tailed ds cDNAs were inserted into the *Pst*I site of pAT153 after poly(dG) tailing¹⁸ of the linearized plasmid.

Transfection of hybrid plasmids into *Escherichia coli* λ1776 (ref. 23) was by the procedure of Hanahan (personal communication). This method yields 3-5 × 10⁷ transformants per µg of supercoiled plasmid DNA. Transfection with recombinant pBR322 (10 ng vRNA-cDNA insert) or pAT153 (10 ng ds cDNA insert) yielded ~500 tetracycline-resistant bacterial colonies. This corresponds to a transformation efficiency of 2 × 10⁴ transformants per µg of tailed, annealed DNA. The colonies from each transformation were picked and replicated to test ampicillin sensitivity. Approximately 60% of the colonies were Tet^r, Amp^r indicating that they contained recombinant plasmids. These plasmids were grown on nitrocellulose filters and analysed by a modification of the *in situ* colony hybridization procedure²⁴ using a ³²P-cDNA probe for influenza

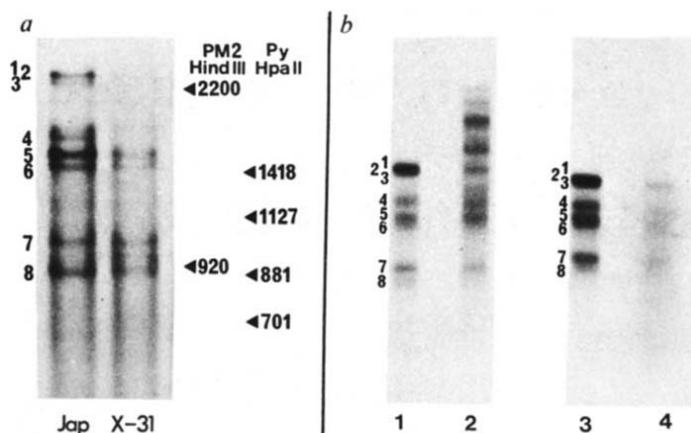


Fig. 1 Characterization of cDNA copies of Japan and X-31 vRNAs. *a*, Separation of cDNA copies of Japan and X-31 vRNAs by agarose gel electrophoresis; polyadenylated vRNAs were reverse transcribed into ss ^{32}P -labelled cDNAs in reaction mixtures containing 50 mM Tris-HCl, pH 8.3, 10 mM MgCl_2 , 140 mM KCl, 30 mM β -mercaptoethanol, 0.5 mM dGTP, 0.5 mM dATP, 0.5 mM TTP, 0.25 mM dCTP ($2 \mu\text{Ci } \mu\text{mol}^{-1}$), $10 \mu\text{g ml}^{-1}$ oligo(dT) 12–18 (P.-L. Biochemicals), 10 units ml^{-1} rat liver ribonuclease inhibitor (Searle), $50 \mu\text{g ml}^{-1}$ polyadenylated vRNA and 360 units ml^{-1} AMV reverse transcriptase (a gift from J. Emtage (Searle)). After incubation for 60 min at 42°C the reaction was terminated by placing the mixture on ice. The cDNAs were separated by electrophoresis on 1.4% agarose gels in 30 mM NaOH, 1 mM EDTA and autoradiographed. The positions of restriction fragments of PM2 (*Hind*III) and polyoma (*Hpa*II) virus DNAs are shown as molecular weight markers. *b*, Electrophoretic resolution of Japan ds cDNAs before and after S_1 nuclease digestion. Single stranded ^{32}P -cDNAs prepared by reverse transcription (lanes 1 and 2) were converted to the ds form (lane 2) and digested with S_1 nuclease (lane 4) by the procedure of Wickens *et al.*²¹. Analysis by electrophoresis on agarose gels was as described in *a*.

sequences prepared by reverse transcription of vRNAs using calf thymus primers as random primers²⁵. 50–80% of the colonies on nitrocellulose filters contained influenza sequences.

To identify which gene segments were represented in the cloned recombinant plasmids, Japan and X-31 vRNAs were separated in agarose gels, transferred to and immobilized on diazotized paper²⁵ and probed by hybridization with ^{32}P -labelled recombinant plasmid DNA prepared by nick translation²⁷. After autoradiography, clones containing the various vRNA genes were identified (Fig. 2). Restriction endonuclease mapping of plasmids which contained HA sequences indicated that clone 2G10 had an insert corresponding to ~95% of the haemagglutinin gene from the Japan strain and clone 4F12 contained an insert corresponding to ~45% of the X-31 HA gene (Fig. 3).

To obtain a clone containing the missing portion of the Japan HA gene, the *Eco*R1-*Pvu*II restriction fragment from the distal end of the 2G10 insert was used to prime synthesis of a cDNA copy from Japan vRNA template. Using the methods described above, a clone (ExB6) containing the missing sequences was identified and mapped by restriction endonuclease digestion (Fig. 3). Finally, a clone (pJHA) containing the entire Japan HA gene nucleotide sequence (except the last 11 nucleotides) was constructed (Fig. 3). Experiments are in progress to use this cloned gene for expression of haemagglutinin protein in prokaryotic and eukaryotic cells.

Determination of the DNA sequence of the cloned inserts was carried out using the procedures of Maxam and Gilbert²⁸. Figure 3 shows the distances sequenced on various restriction fragments. To determine if any of the sequences at the 5' end of the vRNA were not represented in the clones, or if the cloning procedure had resulted in mutations at this end⁸, the 36-base pair *Sau*3A-*Pst*I restriction fragment near the right-hand end of

the ExB6 insert was purified, labelled and used as primer for reverse transcription of vRNA template. The single, labelled DNA product was sequenced and the result indicated that only the last 11 nucleotides of the 3' non-coding region of the Japan vRNA are not represented in the cloned inserts. There were no alterations in the sequence due to the cloning procedure.

Figure 4 provides the completed nucleotide sequences of the complementary RNA strands corresponding to the message coding sequences of the Japan and X-31 genes. Finally, Fig. 6 lists the amino acid sequences derived for the haemagglutinins from Japan, X-31¹¹, 29C¹⁰, Victoria⁹ and Fowl Plague (FP)⁸ viruses.

Structure of the haemagglutinin gene

The Japan haemagglutinin gene is 1,773 nucleotides long. The coding region (1,686 nucleotides) begins with an AUG and ends with an UGA terminator. This is followed in the non-coding region by two in phase and one out of phase UAA triplets. Two of the three reading frames contain frequent termination codons so that only one frame is open for translation. The coding portion of the gene exists as a continuous block of nucleotides. The codon usage is similar to that found for the Victoria⁹ and FPV⁸ haemagglutinin sequences.

There are untranslated regions at both the 5' (43 nucleotides) and 3' (44 nucleotides) ends of the haemagglutinin gene (messenger or cRNA strand). The terminal sequences have been determined previously by direct RNA sequencing^{29,30} or by sequencing of reverse transcripts³¹ and these results confirm that our cloned inserts contain the complete vRNA sequence at the 3' end and miss only 11 nucleotides at the 5' end. The first 14 nucleotides at the 5' end of the HA cRNA and the last 21 nucleotides at the 3' termini are identical in all the strains that have been sequenced^{8–11}. Previous direct RNA sequence analysis of the 5' ends of vRNA and cRNA transcripts of the eight influenza gene segments from a number of different viral strains showed that, in all cases, the terminal 12–13 nucleotides are closely conserved and that the 5' and 3' ends display inverted partial complementarity²⁹. This structure may have a role in the process of viral replication. Emtage *et al.*³² have shown that there is an adventitious prokaryotic-like ribosome binding site near the 5' end of the cRNA which may be functional during expression of the cloned gene in *E. coli*. This sequence is also

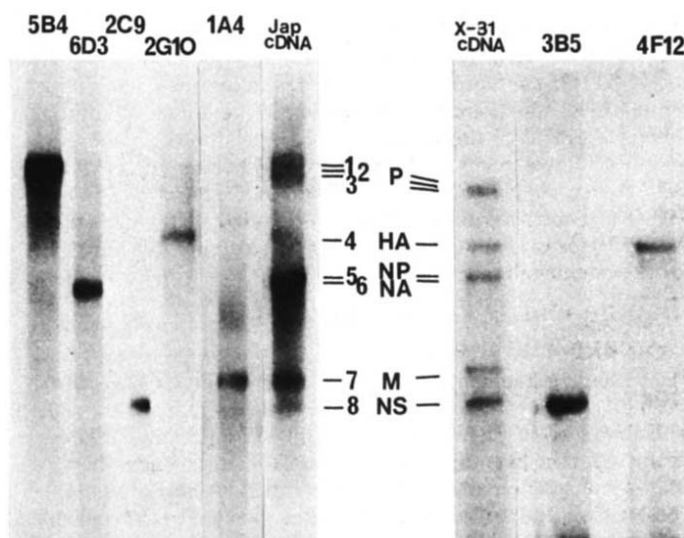
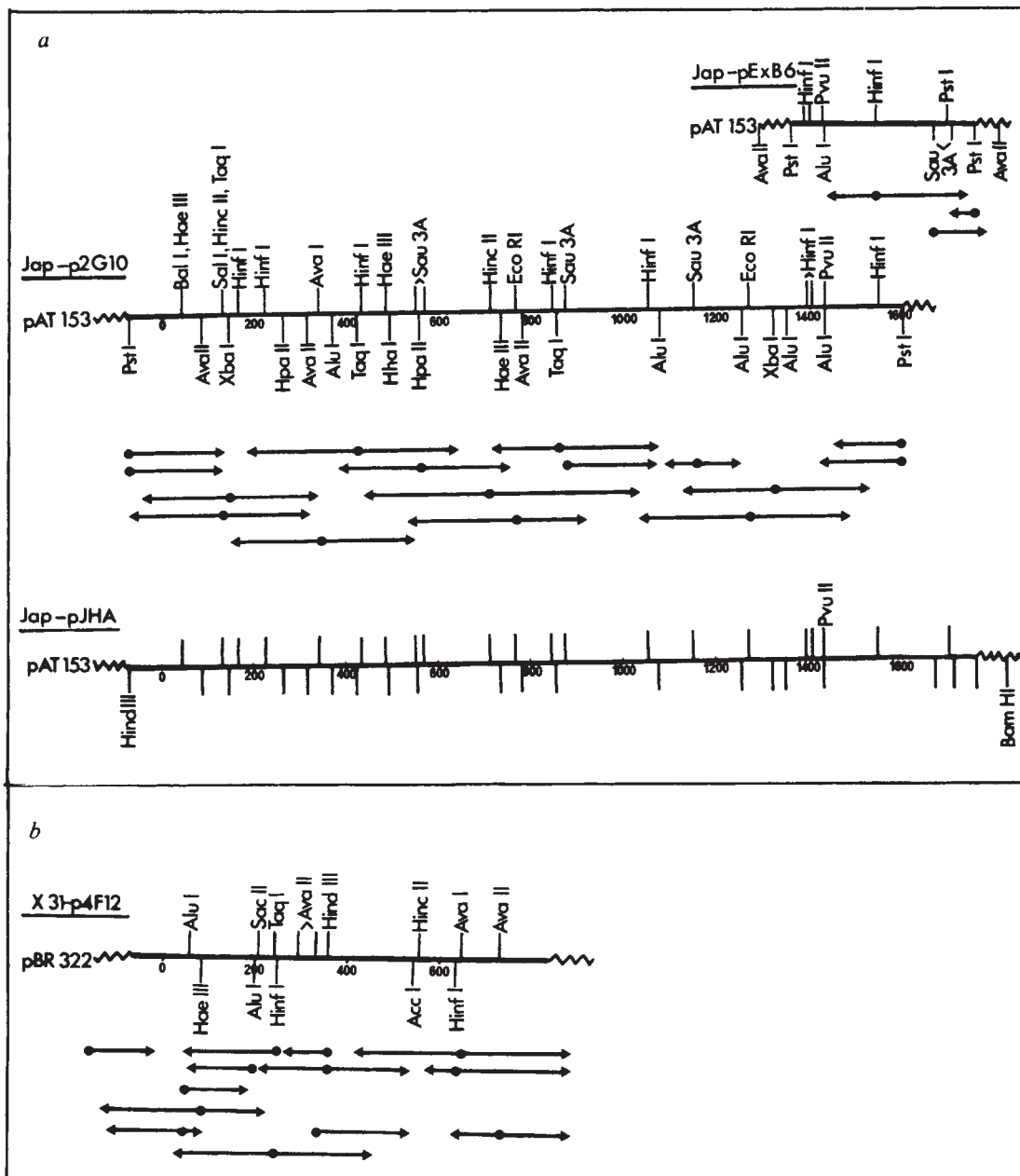


Fig. 2 Assignment of cloned inserts to viral RNA segments. Recombinant plasmid DNA preparations from the numbered clones were labelled with ^{32}P by nick translation²⁷ and hybridized to fractionated vRNAs immobilized on diazotized paper²⁶. The positions of the eight vRNA genes were identified by hybridization with the appropriate ^{32}P -labelled cDNAs prepared by reverse transcription of total X-31 or Japan vRNAs²⁵.

Fig. 3 Restriction enzyme maps of *a*, Japan and *b*, X-31 haemagglutinin gene inserts in recombinant plasmids. The construction of clones p2G10, pExB6 and p4F12 is described in the text. Conditions for all restriction enzyme digestions were as described in the catalogue of New England Biolabs. The distances and directions sequenced on various restriction fragments are shown by arrows. Clone pJHA contains a copy of the entire Japan HA gene except for the last 11 nucleotides of the 5' non-translated region of the vRNA. It was constructed from the p2G10 and pExB6 clones as follows: the gene insert in p2G10 was excised with *Pst*I and *Hind*III linkers were attached to the ends⁵². The *Hind*III-*Pvu*II restriction fragment was purified. Similarly the gene insert in pExB6 was excised by cutting the flanking pAT153 sequences with *Ava*II and *Bam*HI linkers were attached to the ends⁵² before purification of the *Pvu*II-*Bam* restriction fragment. The *Hind*III-*Pvu*II and *Pvu*II-*Bam* fragments were then ligated between the *Hind*III and *Bam*HI sites of pAT153 using T4 DNA ligase. After transfection of the ligated mixture into χ 1776 a clone pJHA containing the composite full-length insert was obtained and characterized.



present in the human influenza HA genes but the greater distance between it and the AUG codon suggests that it may not function in expression of these genes in bacteria³³. The remainder of the untranslated regions vary in length and in sequence and show little evidence of common origin or function.

Structure of the haemagglutinin protein predicted from the nucleic acid sequence: cleavage of the polypeptide

The haemagglutinin glycoprotein is synthesized in infected cells as a single polypeptide precursor which is converted by proteolytic cleavage into two disulphide-bonded polypeptides HA1 and HA2^{34,35}. The Japan haemagglutinin precursor consists of 547 amino acids and a single arginine residue is removed during formation of HA1 (324 amino acids) and HA2 (222 amino acids)⁶. In this respect Japan haemagglutinin differs from FPV haemagglutinin⁸ as at the equivalent site there is a sequence of six charged amino acids which are probably removed during processing. The difference in structure at this site may explain why FPV is susceptible to cleavage activation in a wider range of host cells³⁶.

Hydrophobic regions of the haemagglutinin

The hydrophobic sequence at the new NH₂ terminus of HA2 which is generated by this cleavage is highly conserved in influenza haemagglutinins³⁷ and also shows a high degree of similarity to the NH₂ terminus of the F1 subunit of Sendai virus fusion protein⁴, which is revealed after cleavage activation of infectivity and fusion activity. Previously we have suggested that these sequences may have a functional role in interaction with and penetration of the cell membrane during infection.

The HA primary translation product contains two additional hydrophobic regions which are present in all the virus strains. These are the signal sequence at the NH₂ terminus and the presumed membrane insertion sequence at the COOH terminus. The nascent Japan HA polypeptide chain contains a hydrophobic signal peptide^{6,30,38} which is probably involved in the transport of the polypeptide across the endoplasmic reticulum. This peptide is cleaved off when the process of membrane transfer and insertion is complete. The DNA sequence reported here defines a 15-residue peptide containing 14 uninterrupted non-polar amino acids before the known NH₂ terminus of HA1⁶ and agrees with the data of Air³¹ and with our previous data from direct RNA and amino acid sequence analyses³⁰. The H3 haemagglutinins⁹⁻¹¹ probably also have a

signal peptide of 16 amino acids containing 14 uninterrupted non-polar residues since the glutamine residue at position 17 is probably the NH₂-terminal amino acid of the mature protein⁷. Again, although amino-acid sequence data is not available for FPV, homologies at the NH₂ terminus of HA1 suggest that FPV haemagglutinin has an 18 amino-acid signal peptide containing a core of 11 hydrophobic amino acids⁸. It is striking that the proposed signal peptides of the haemagglutinins from the H2, H3 and avian subtypes show no homology with each other, indicating that it is the hydrophobicity and not specific sequence that is important for membrane association. The size and non-polar character of these prepeptides are similar in other membrane or secreted proteins³⁹.

The third hydrophobic sequence at the COOH terminus of the primary translation product and of HA2 is probably involved in anchoring the protein in the lipid envelope³⁷. The haemagglutinins from the H2, H3⁹⁻¹¹ and FPV⁸ strains have 41, 27 and 26 non-polar amino acids, respectively, preceding charged amino acids near the COOH terminus. There is some similarity between these sequences but it is significantly less than that found in the rest of HA2. It may be that, as in the case of the signal peptide, it is hydrophobicity and not specific sequence that defines the interaction with the membrane. It is of course not possible to define from these data which amino acids are embedded in the lipid bilayer, but it seems likely that there are no more than 10-12 residues on the inner face of the virus envelope, suggested by the location of charged amino acids at position 10 (H3)⁹⁻¹¹ and 11 (FPV)⁸ amino acids from the COOH terminus. The only charged amino acid at the COOH terminus of Japan HA2 is four residues from the end. Similar numbers of hydrophobic amino acids span the lipid bilayer in HLA heavy chain⁴⁰ and glycoporphin A⁴¹. However, in these cases the internal hydrophilic tails (approximately 30 residues) are longer.

Conservation of amino acid sequence and location of cysteine or cystine residues

It is now possible to compare the complete amino acid sequence of haemagglutinins from two subtypes of human influenza virus and an avian virus⁸⁻¹¹. In Fig. 5, the protein sequences have been aligned using a computer program⁴² to optimize the homology. The alignment involves only a few small insertions or deletions in the sequences, except at the NH₂ terminus of HA1 where the H3⁹⁻¹¹ haemagglutinins have 10 or 11 extra amino acids and at the cleavage site between HA1 and HA2 where the single arginine residue in the human sequences is replaced by six charged amino acids in the avian FPV haemagglutinin. As shown in Fig. 5 and Table 1, there is considerable sequence

Table 1 Conservation of amino acids in haemagglutinin sequences

Amino acids conserved in haemagglutinin sequences of	HA No. (%)	HA1 No. (%)	HA2 No. (%)
All 3 strains (H2, H3, Hav1)	165 (30)	72 (22)	93 (42)
H2 (Japan) and H3 (Victoria)	229 (42)	118 (36)	111 (50)
H2 (Japan) and Hav1 (FPV)	235 (36)	118 (36)	117 (53)
H3 (Victoria) and Hav1 (FPV)	263 (48)	119 (36)	144 (65)
Total	547	325	222

homology between the haemagglutinins of all three subtypes (30%). The similarity is even more marked between H2 and Hav1 haemagglutinins (43%), H2 and H3 haemagglutinins (42%) and Hav1 and H3 haemagglutinins (48%). Within the H3 subtype there is 95% sequence homology between the 1968 (X-31) and 1975 (Victoria) isolates. There is a much greater degree of conservation in HA2 than in HA1. Although there can be extensive variation in amino acid sequence (especially in HA1), the results of these observations on the constant size of the haemagglutinins and comparative studies of their secondary structure⁴³ suggest that all haemagglutinins have a similar three-dimensional structure. This idea is emphasized by the striking conservation of the location of the cysteine residues (Fig. 6) which is probably due to constraints on the folding of the molecule and formation of disulphide bridges. The only portion of the molecule in which there is any variation in the location of the sulphhydryl residues is at the COOH terminus where the H3⁹⁻¹¹ and Hav1 haemagglutinins have more cysteines than the H2 protein. Since these residues are probably embedded in the lipid bilayer, their positions may not be constrained by the requirement for formation of disulphide bonds. We have shown that in Japan haemagglutinin all 12 cysteine residues in the external protein spike are involved in disulphide bridges, of which only one (Cys 4-Cys 462) is an interchain link between HA1 and HA2 (unpublished results). The secondary structure formed by the remaining three cysteines, which are either in the lipid bilayer or on the inner side of the membrane, has not yet been elucidated. It is clear, however, that although the COOH terminus, which may be on the inner side of the membrane, could interact with the viral matrix protein, the two cysteine residues at positions 2 and 5 from the terminus of HA2 do not form disulphide bonds linking the two proteins.

Glycosylation of the haemagglutinins

The haemagglutinin molecule is glycosylated during transport from the endoplasmic reticulum to the plasma membrane of the infected cell⁴⁴. Figure 6 shows the positions of the predicted

JAP cRNA 5'-AGCAAAGCAGGGGUAUACCAUAGACACCAAGCAAAACA-----AUG GCC AUC AUU UAU CUC AUU CUC CUG₆₀
 X31 cRNA 5'-AGCAAAGCAGGGGUAUACCAUAGACACCAAGCAAAACA-----AUG GCC AUC AUU UAU CUC AUU CUC CUG₆₀
 JAP UUC ACA GCA GUG AGA GGG GAC CAG UAU UGC AUU GGA UAC CAU GCC AAU AAU UCC ACA GAG AAG GUC GAC ACA AAU CUA GAG CGG AAC GU₆₀
 X31 GAA AAU GAC AAC GAC ACG UGC GUG GGA CAU CAU GCG GUG CCA AAG GGA ACA CUA GUG AAA ACA AUC ACA GAU GAU CAG UAU
 JAP ACU GUG ACU CAU GCC AAG GAC AUU CUU GAG AAG ACC CAU AAC GGA AAG UUA UGC AAA CUA AAC GGA AUC CCU CCA CUU GAA CUA GGG GAC₂₆₀
 X31 GAA GUG ACU AAU GCU ACG GAG CUA GUU CAG AGC UCC UCA ACG GGG AAA AUA UGC AAC --- AAU CCU CAU CGA AUC CUU GAU GGA AUA GAC
 JAP UGU AGC AUU GCC GGA UGG CUC CUU GGA AAU CCA GAA UGU GAU AGG CUU CUA AGU GUG CCA GAA UGG UCU UUA AUG GAG AAA GAA AAC₃₄₀
 X31 UGC ACA CUG AUA GAU GCU CUA UGU GGG GAC CCU CAU UGU GAU --- GUU UUU CAA AAU GAG ACA UGG GAC CUU UGU UUU GAA CGC AGC AAA
 JAP CCG AGA GAC GGU UGU UAU CCA GGC AGC UUC AAU GAU UAU GAA GAA UUG AAA CAU CUC CUC AGC AGC GUG AAA CAU UUC GAG AAA GU₄₀₀
 X31 GCU UUC AGC AAC --- UGU UAC CCU AAU GAU GUG CCA GAU UAU GCC UCC CUU AGG UCA CUA GUU GCC UCG UCA GGC ACU CUG GAG UUU AUC
 JAP AAG AUU CUG CCC AAA GAU AGA UGG ACA CAG CAU ACA ACA ACU GGA GGU UCA CGG GCC UGC GCG GUG UCU GGU AAU CCA UCA UUU UUC AG₆₂₀
 X31 ACU GAG GGU UUC ACU --- UGG ACU GGG GUC ACU CAG AAU GGG GGA AGC AAU GCG UGC AAA AGG GGA CCG GGU AGC GGU UUU UUC AGU
 JAP AAC AUG GUC UGG CUG ACA AAG GAA GGA ACA GAU UAU CCG GUU GCC AAA GGA UCG UAC AAC AAU ACA AGC GGA GAA CAA AUG CUA AUA AU₆₆₀
 X31 AGA CUG AAC UGG UGG AAC AAA GCA GCA ACA UAU CCA CUG CUG AAC GUG ACU UGC ACA AAC AAU CAA UUU GAC AAA CUA UAC AUU
 JAP UGG GGG GUG CAC CAU CCC AUU GAU GAG ACA GAA CAA ABA ACA UUG UAC CAG AAU GUG GGA ACC UAU CUU UCC GUA GGC ACA ACA UUG₇₀₀
 X31 UGG GGG AUU CAC CAC CCG AGC AGC AAG CAA GAA CAA ACC AEC CUG UAU GUU CAA GCA UGA GGG AGA GUC ACA CUC UGU ACC AGG AGA AGC
 JAP AAC AAA AGG UCA ACC CCA GAA UUA GCA ACA AGG CCU AAA GUG AAU GGA CAA GGA GGU AGA AUG GAA UGU UGU ACC CUC UGU GAU AU₇₉₀
 X31 CAG CAA ACU AUA AUC CCG AAU AUC GGG UCC AGA CCC UGG UUA AGG GGU CUG UCU AGU AGA AUA AGC AUC UAU U₇₇₇
 JAP UGG GAC ACC AUA AAU UUU GAG AGU ACU GGU AAU CUA AUA CCA GAG UAU GGA UUC AAA AUA UCG AAA AGA GGU AGU UCA GGG AUC AU₈₈₀
 JAP AAA ACA GAA GGA ACA CUU GAG AGU GAG ACC AAA UGC CAA ACU CCU UUG GGA GCA AUA AAU ACA ACA UUG CCU UUU CAA AAC GUC CAC₉₇₀
 JAP CCA CUG ACA AUA GGU GAG UGC CCC AAA UAU GUA AAA UCG GAG AAG UUG GUC UUA GCA ACA GGA CUA AGG AAU GUU CCC CAG AUU GAA UCA₁₀₆₀
 JAP AGA GGA UUG UUU GGG GCA AUA GCU GGU UUU AUA GAA GGA GGA UGG CAA GGA AUG GUU GAU GGU UGG UAU GGA UAC CAU CAC AGC AAU GAC₁₁₅₀
 JAP CAA GGA UCA GGG UAU GCA GAC GAA AAA GAA UCC ACU CAA AAG GCA UUU GAU GGA AUC ACC AAC AAG GUA AAU UCU GUG AUU GAA AAG AU₁₂₄₀
 JAP AAC CCA UUU GAA GCU GUU GGG AAG GAG AUC GGU AAC UUA GAG AGA CUG GAG AAC UUG AAC AAA AGG AUG GAA GAC GGG UUU CUA₁₃₃₀
 JAP GAU GUG UGG ACA UAC AAU GCU GAG CUU CUA GUU CUG AUG GAA AAU GAG AGG ACA CUU GAC UUU CAU GAU UCU AAU GUC AAG AAU CUG UAU₁₄₂₀
 JAP GAU AAA GUC AGA AUG CAG CUG ACA GAC AAC GUC AAA GAA CUA GGA AAU GGA UGU UUU GAA UUU UAU CAC AAA UGU GAU GAA UGC AUG₁₅₁₀
 JAP AAU AGU GUG AAA AAC GGG ACG UAU GAU UAU CCC AAG UAU GAA GAA GAG UCU AAA CUA AAU AGA AAU GAA AUC AAA GGG GUA AAA UUG AGC₁₆₀₀
 JAP AGC AUG GGG GUU UAU CAA AUC CUU GCC AUU UAU GCU ACA GUA GCA GGU UCU CUG UCA CUG GCA AUC AUG AUG GCU GGG UUC UUC UG₁₆₉₀
 JAP AUG UGC UCC AAC GGG UCU CUG CAG UGC AGG AUC UGC AUA UUA UUA GUCAUUUUAUAUAUAACACCCUUGUUCUACU-3'
 1773

Fig. 4 Complementary RNA sequences of haemagglutinin genes derived by sequence analysis of the cloned gene inserts in plasmids 2G10, ExB6 and 4F12.

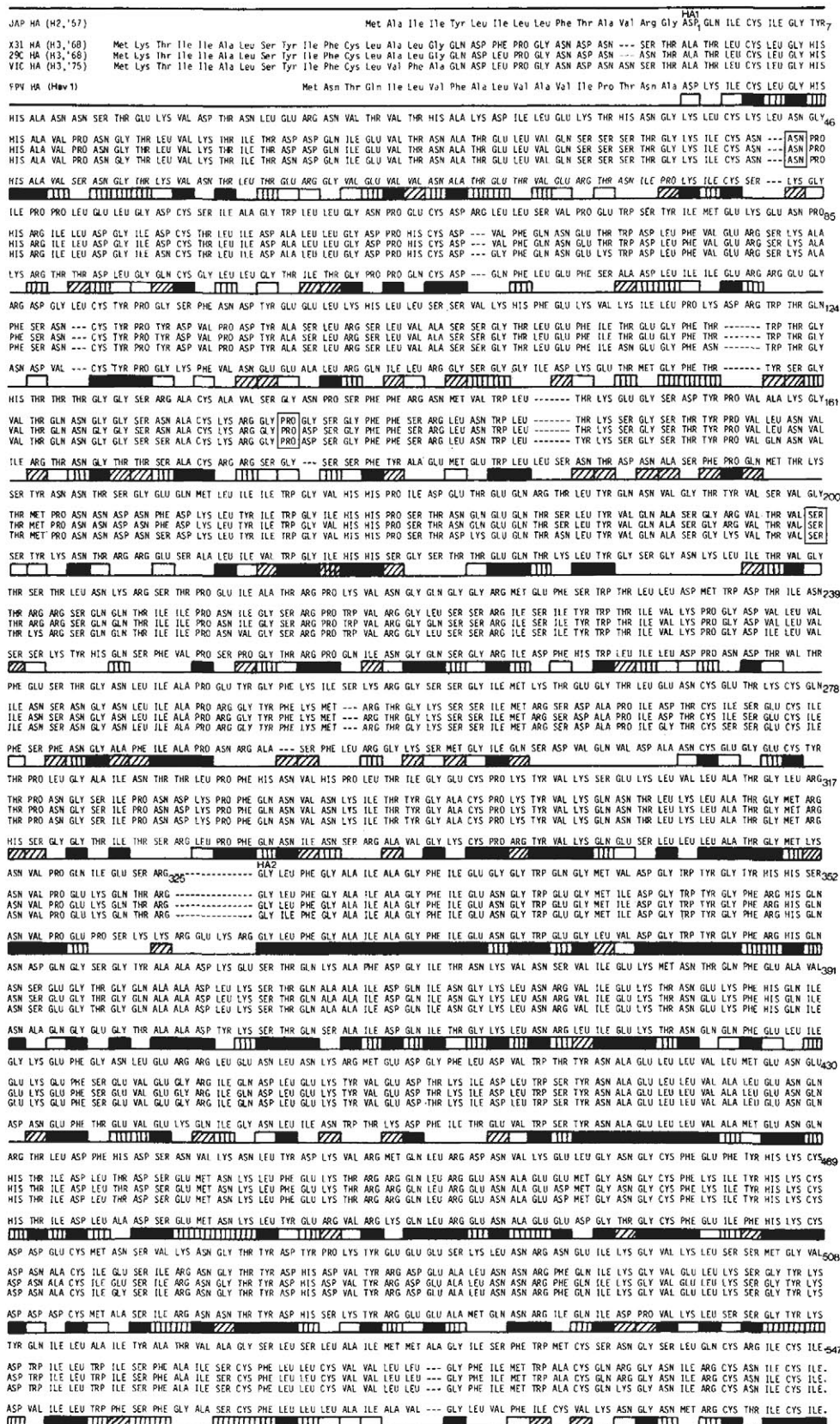


Fig. 5 Amino acid sequences of influenza haemagglutinins. The amino acid sequences of the Japan (H2) and X-31 (H3) haemagglutinins are compared with the sequences from other strains: Victoria (H3)⁹ NT60/29C (H3)¹⁰ and Fowl Plague Virus (Hav1)⁸. The COOH terminal amino acid sequence of the X31 haemagglutinin which was not determined by us is from Verhoeven *et al.*¹¹. The sequences have been aligned to maximize homology which is shown between ■, H2, H3 and Hav1 proteins; □, H2 and Hav1 proteins; and ▨, H3 and Hav1 proteins.

carbohydrate attachment sites Asn-X-Ser or Thr⁴⁵ in the haemagglutinin sequences. We have confirmed that five of the six possible sites on Japan haemagglutinin are indeed glycosylated (unpublished results). The absence of carbohydrate at the remaining site (Asn₁₃₀-Pro-Ser) is consistent with reports that such a sequence is not glycosylated in other proteins⁴⁵. It remains to establish whether all the other possible sites in the H3 and Hav1 haemagglutinins are in fact glycosylated. Although

there is some conservation of the number and position of the carbohydrate residues, there is clearly some latitude for variation in their location. As shown in Fig. 6, there is even variation in the possible attachment sites between haemagglutinins of the H3 subtype. A possible constraint would be a requirement that the carbohydrate should be on the outside of the molecule. Confirmation of such a constraint awaits analysis of the three-dimensional structure of the molecule⁴⁶.

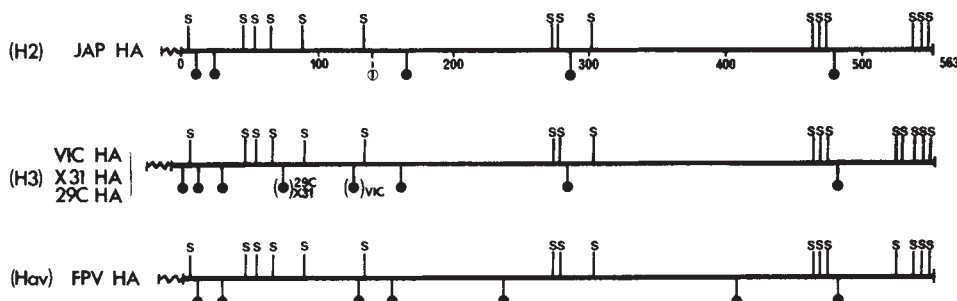


Fig. 6 Schematic representation of the positions of cysteine residues and glycosylation sites in the haemagglutinin amino acid sequences. |, Cysteine residue; |, carbohydrate side chain;

Antigenicity, shift and drift

The greater degree of variation in HA1 than in HA2 is consistent with the observations^{47,48} that the major antigenic sites are located in HA1 and that it is variation in these sites which allows the virus to escape from neutralizing antibody. Between the major shifts in antigenic structure which define the beginning and end of each pandemic era, the haemagglutinin undergoes a series of changes, due to the pressure of increasing antibody prevalence in the population, known as antigenic drift.

The changes responsible for antigenic drift accumulate with time and field strains isolated several years apart from within a single pandemic era (for example, H3:1968 to present) show a large number of amino acid changes in peptide mapping studies⁴⁹. In this study we can deduce additional characteristics of the process of antigenic drift by comparing the nucleic acid and protein sequences of the haemagglutinins of the H3 subtype. Clearly, drift occurs by a simple stepwise mutation of the HA nucleotide sequence. There are 65 nucleotide differences between the X-31 (ref. 11) (1968) and Victoria⁹ (1975) haemagglutinin genes, resulting in 28 amino acid changes. Although all but one of the amino acid changes could have occurred via single base changes, in five cases double mutations have occurred. The two 1968 isolates (X-31 and the laboratory variant NT60/29C) differ by eight nucleotides and six amino acids. In four cases, the new amino acid is the same as that in the Victoria protein. Similarly, of the 482 amino acids known from the protein chemistry analysis of the haemagglutinin from the 1972 isolate A/Memphis/102/72 (refs 7, 53), 21 amino acids differ in the X-31 protein, although 12 of these amino acids are the same in the NT60 or Victoria haemagglutinins.

It is likely that some of the changes which occur in the haemagglutinin may be antigenically neutral or may cause antigenically significant alterations in distant parts of the protein. So far, it has not been possible to correlate the locations of antigenic sites with the positions of amino acid substitutions. Indeed, the variation within subtypes in a pandemic series is so major that nucleic acid and/or amino acid sequence information from even a large number of field isolates would not provide unequivocal information on the antigenic sites. To simplify this system other workers have used monoclonal antibodies directed against antigenic sites in HA to select variants *in vitro* and thus to mimic antigenic drift⁵⁰. Such studies have so far indicated three or four regions of the HA1 which may constitute antigenic sites. The positions which change in these variants are boxed in Fig. 5.

The more dramatic changes which constitute an antigenic shift are illustrated by a comparison of the amino acid sequences of the H2 and H3 pandemic subtypes (Fig. 5). Clearly the H3 haemagglutinin cannot have arisen by stepwise mutation of the gene of the previous H2 pandemic type and they are so different that it is impossible to map the various antigenic sites by comparing their primary sequences. Furthermore, the fact that the haemagglutinins of the two human strains are no more similar to each other than to that of the avian strain makes it impossible to establish a genealogical tree which would define the evolutionary relationship between them. Recombination or reassortment of haemagglutinin genes between influenza viruses of humans and those of domestic or wild animals has been suggested as the mechanism of antigenic shift⁵¹. The data presented here are not inconsistent with such a theory.

Hybrid plasmids were transfected into and propagated in *E. coli* χ 1776 in containment conditions approved by the British Genetic Manipulation Advisory Group.

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LETTERS

Massive black hole binaries in active galactic nuclei

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Most theoretical discussions of active galactic nuclei (including quasars) attribute their energy production either to an accreting black hole or to a precursor stage—for instance a dense star cluster or a supermassive star—whose inevitable end point is a massive black hole¹. We explore here the possibility that some active nuclei may contain two massive black holes in orbit about each other. This hypothesis suggests a new interpretation for the observed bending² and apparent precession³ of radio jets emerging from these objects and may indeed be verified through detection of the direct consequences of orbital motion.

There are straightforward reasons for surmising that supermassive binaries exist^{4,5}: mergers between galaxies appear to be frequent; cD galaxies in clusters and small groups were quite probably formed in this manner⁶⁻⁹; and there is direct evidence that the nearby active galaxy Centaurus A is a merger product⁹. If most galaxies contain massive black holes in their nuclei—relics of earlier activity—then they will settle, under dynamical friction, in the core of a merged stellar system. Many cD galaxies do indeed contain double or multiple cores that may have been formed in this way¹⁰. There are other scenarios for binary black hole formation. For instance, a rotating super-massive star may undergo bar-mode instability and fission into two components that both collapse¹.

The dynamical evolution of a pair of massive black holes in the violently relaxed core of a newly merged galaxy proceeds through several stages.

(1) The black holes are probably surrounded by dense stellar clusters; that within the less massive galaxy should be denser and will survive intact until the nuclei merge. Dynamical friction is extremely efficient⁶ and the cores merge and undergo violent relaxation in a characteristic galactic dynamical time $t_{\text{gal}} \sim 10^8$ yr.

(2) The captured hole sinks toward the centre of the stellar distribution on the dynamical friction time scale:

$$t_{\text{df}} \sim \frac{6 \times 10^6}{\log N} \left(\frac{v_c}{300 \text{ km s}^{-1}} \right) \left(\frac{r_c}{100 \text{ pc}} \right)^2 m_8^{-1} \text{ yr} \quad (1)$$

where the core is presumed to have a central velocity dispersion v_c , a core radius r_c and contain N stars, mass m^* . m_8 is the mass of the smaller black hole, in units of $10^8 M_\odot$, presumed to be associated with the smaller galaxy. M is the mass of the larger black hole, assumed to be less than Nm^* (the mass of the core). t_{df} is generally longer than the core crossing time and so the merged cores should be fairly relaxed.

(3) When the black hole separation r shrinks to a value $r_B \sim (M/Nm_*)^{1/3} r_c$, they will become bound to each other with an orbital velocity that is initially smaller than the velocity dispersion of the stars by a factor $(M/Nm_*)^{1/3}$.

(4) The holes continue to spiral together under dynamical friction. As the binary becomes 'harder', the maximum impact parameter for effective energy transfer diminishes as $(r/r_B)^{3/2} r_c$ because a binary cannot lose energy effectively to a field star during an encounter which lasts longer than one orbital period. For longer encounter times, the orbital energy is adiabatically

invariant and the energy transfer diminishes exponentially¹¹. This affects t_{df} only through the logarithm as long as the binding energy of the binary is small compared with the total kinetic energy of all the stars whose orbits pass sufficiently close to interact effectively.

(5) When $r \leq r_{\text{lc}} = (m/M)^{1/4} (r_B/r_c)^{9/4} r_c$, this condition is violated and further stellar dynamical evolution is controlled by the rate at which stars whose orbits do not come close to the binary can diffuse into more radial 'loss cone' orbits¹² that pass within a radius $\sim r$. Two-body 'star-star' relaxation will repopulate the loss cone in a time $\sim$ (solid angle of loss cone) $\times$ (two-body relaxation time). The loss cone must be repopulated $(r_{\text{lc}}/r)^4$ times to halve the binary orbit. The relevant binary evolution time is then:

$$t_{\text{lc}} \sim t_{\text{df}} \max \left[1, \left(\frac{m}{M} \right)^{7/4} N \left(\frac{r_B}{r_c} \right)^{27/4} \left(\frac{r_{\text{lc}}}{r} \right) \right] \quad (2)$$

Collective effects may, however, lead to a more rapid repopulation of the loss cone¹³ and evolution on a time closer to t_{df} .

(6) The binary becomes 'hard' when its orbital speed reaches v_c at a separation $r_h \sim (r_B/r_c)^3 r_c$. The maximum impact parameter for dynamical friction is now $(rr_h)^{1/2}$. Each interacting star on average carries off a fraction $\sim m_*/M^2$ of the binary's binding energy. The binary evolution time scale is now

$$t_h \sim t_{\text{df}} \max \left[\left(\frac{r_h}{r} \right) \ln N, \left(\frac{M}{Nm^*} \right)^2 N \right] \quad (3)$$

(7) When the binary becomes sufficiently tightly bound, gravitational radiation shrinks the orbit on a time scale $|r/\dot{r}|$ given by

$$t_{\text{GR}} \sim 3 \times 10^5 \left(\frac{m}{M} \right)^{-1} M_8^{-3} r_{16}^4 \text{ yr} \quad (4)$$

(8) The evolution now proceeds quite rapidly to coalescence (which takes a time $\sim M_8$ h). This may be accompanied by a powerful burst of electromagnetic radiation, depending on the local gas density and magnetic field strength, as well as a pulse of gravitational radiation^{4,5}. Indeed, the final plunge will lead to the emission of net linear momentum and the recoil may be sufficient to eject the combined hole from the core and hence from the galaxy⁵.

In addition to these stellar dynamical effects, infall of gas onto the binary can also lead to some orbital evolution. Gas may be flung out of the system, acquiring energy (and angular momentum) at the expense of the binary; alternatively, gas may accrete onto the larger hole, causing orbital contraction as the product Mr is adiabatically invariant. In either case, the evolution time scale is

$$t_{\text{gas}} \sim 10^8 M_8 (\dot{M}/1 M_\odot \text{ yr}^{-1})^{-1} \text{ yr} \quad (5)$$

During phases of nuclear activity, gas infall rates $\dot{M}$ of $1-10 M_\odot \text{ yr}^{-1}$ are required for times 10^7-10^8 yr. It is, therefore, quite likely that this process controls the binary evolution when r just exceeds r_{GR} and the stellar dynamical effects are weakest.

These time scales, $|r/\dot{r}|$, are given schematically in Fig. 1. It is clear that the longest evolutionary phase, and therefore the one containing most potentially observable binaries, is that with $r_h \geq r \geq r_{\text{GR}}$. During this phase a tight binary will persist in a quiescent state with almost constant r , except when it is undergoing fuelling and it becomes a conspicuous source of non-thermal power. The energy dissipated by the material flung out (at the expense of the binary orbital energy) is generally negligible at this time¹⁴. Slow secular contraction proceeds during active phases and pauses during intervals of quiescence. The details and statistics of observability depend on the time-dependence of $\dot{M}$ (is it intermittent or recurrent?, are the active phases as long as $M/\dot{M}$?). In conditions appropriate to galaxies like M87 ($r_c \sim 100$ pc, $v_c \sim 300$ km s⁻¹, $Nm^* \sim 3 \times 10^9 M_\odot$) it is

clear that the two-body evolution times given by equations (2) and (3) are much longer than t_{gas} . (This may not be true for much less massive cores and small holes, $M \leq 10^6 M_\odot$.) If the binary gains mass at a constant rate, then the probability of observing a binary with separation greater than r will fall as r^{-1} . If $M \propto \dot{M}$, then binaries are distributed uniformly in $\log r$. In any case, the separation r_{GR} at which gravitational radiation becomes dominant is insensitive to the values of t_h and t_{gas} ,

$$r_{\text{GR}} = 5 \times 10^{16} \left(\frac{m}{M} \right)^{1/4} M_8^{3/4} \times \left[\frac{\min(t_h, t_{\text{gas}})}{10^8 \text{ yr}} \right]^{1/4} \text{ cm} \quad (6)$$

For $r \leq r_{\text{GR}}$ the orbital evolution is very rapid and the probability of observing a binary with separation smaller than r will decline as r^4 . Most observed binaries will have $r_{\text{GR}} \lesssim r_h$.

The most direct evidence for a massive black hole binary would be some manifestation of the keplerian motion, which has a period

$$P_{\text{orb}} \sim 1.6 r_{16}^{3/2} M_8^{-1/2} \text{ yr} \quad (7)$$

Any eccentricity in the orbit will almost certainly be erased by the frictional drag and so the orbit is assumed to be circular. We generally expect the spin axes of the holes to be misaligned unless they have both accreted a substantial amount of gas with a fixed angular momentum¹⁵. The spin axes will then undergo geodetic precession about the total angular momentum. For the more massive hole, the period is,

$$P_{\text{prec}} \sim 600 r_{16}^{5/2} (M/m) M_8^{-3/2} \text{ yr} \quad (8)$$

The spin angular momentum will be small compared with that in the binary provided that $(m/M) \gg (GM/rc^2)^{1/2}$ and so the spins precess in a cone with the orbital angular momentum as axis.

The beams of plasma which give rise to compact and extended radio structure in active galaxies may be collimated sufficiently near a massive black hole that the Lense-Thirring effect aligns them with the hole's axis¹⁵, irrespective of the original momentum of the inflowing gas. (This requires the collimation scale to be much smaller than the binary separation r in the phases that concern us here.) The beams will then precess with period P_{prec} . It has been suggested that a scaled-down version of this mechanism causes a pair of jets to precess in SS433 (refs 16, 17).

As (r_h/r_{GR}) can be as large as 10^4 , P_{orb} and P_{prec} can span many orders of magnitude and be made manifest in different ways. We outline below two examples that could correspond to different phases in the evolution of a binary. The observed activity is assumed to be only associated with the more massive component.

Wide binary ($r_{16} \sim 30$, $M_8 \sim 1$, $m_8 \sim 0.1$). In this case the precession time scale is $\sim 3 \times 10^7$ yr—comparable with the inferred lifetime of extended radio components. Precession of the beam axis would then result in an inversion symmetry in the source structure. Such precession has been inferred³ on phenomenological grounds, but the massive binary seems to be the only way to set this in a dynamical context. (Jet alignment can occur, however, with a lone black hole¹⁵.) Note that the morphology may differ from that associated with a ballistic trajectory, because of gas-dynamical effects, but should nevertheless have the same average symmetry (M.C.B., in preparation).

The orbital period (300 yr) would be too long to be detected directly. However, the broad emission lines may originate in a region around the more massive hole while the narrow lines would come from a much larger system enveloping the binary. The broad lines may then peak at a velocity up to $\sim 10,000 r_{16}^{1/2} M_8^{-1/2} m_8 \text{ km s}^{-1}$ different from that of the galaxy as determined by the narrow lines. Blue- and redshifts (relative to the narrow lines) should be about equally common.

Close binary ($r \sim r_{\text{GR}}$, $t_{\text{gas}} \sim 10^8 \text{ yr}$). In this case $P_{\text{orb}} \sim 18(m/M)^{3/8} M_8^{5/8} \text{ yr}$, $P_{\text{prec}} \sim 3 \times 10^4 (M/m)^{3/8} M_8^{3/8} \text{ yr}$. Any extended radio structure associated with jets, built up over many

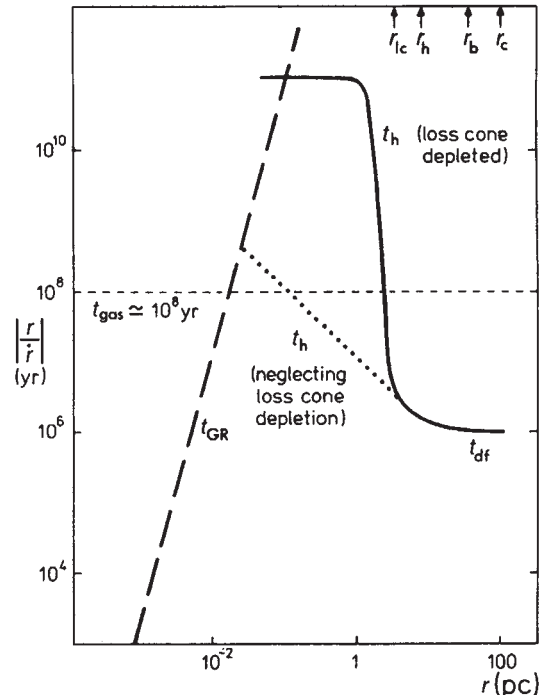


Fig. 1 Diagram of the time scales involved in the approach, and eventual coalescence, of a supermassive binary. The core parameters chosen are those that might be appropriate to a giant elliptical: $r_c \sim 100 \text{ pc}$, $v_c \sim 300 \text{ km s}^{-1}$, $N \sim 2 \times 10^9$ and $m_8 \sim 1$. The members of the binary are taken to have masses $M_8 = 1$ and $m_8 = 0.3$. For this system, $t_{\text{df}} \sim 10^6 \text{ yr}$. Within r_h the evolution time scale would be $(r_h/r)t_{\text{df}}$ if loss cone depletion could be ignored; however, unless collective effects permit replenishment of the loss cone on much less than the ordinary stellar relaxation time, the evolution within r_c proceeds on a very much longer time scale $\geq 10^{11} \text{ yr}$ (compare equation (3)). Influx of gas into the system at a rate $\dot{M} \sim 1 M_\odot \text{ yr}^{-1}$ would however yield a time scale $\sim 10^8 \text{ yr}$. Gravitational radiation would take over as the dominant mechanism within $1.3 \times 10^{-2} (t_{\text{gas}}/10^8 \text{ yr})^{1/4} \text{ pc}$. The values of the various critical radii are indicated. (Note that in this particular example r_c lies inside r_h ; for the case of two similar masses, as discussed in the text, this ordering is reversed.)

precession periods, will be aligned with the orbital angular momentum; the precession related curvature will be discernible, however, on the scales probed by VLBI or the VLA.

Suppose that the jets precess around a cone of semi-angle ϕ . If the jets were narrow with a non-relativistic speed $V \ll c$, a 'snapshot' would reveal the beam material tracing out a spiral pattern around the core: successive turns would be separated by a fixed distance vP_{prec} , the pattern becoming more transverse at larger distances from the apex. However, in the 'superluminal' sources the jet velocity is believed to be relativistic with Lorentz factor $\gamma = (1 - v^2/c^2)^{-1/2}$ in the range 5–10 (ref. 2). What is actually observed from a precessing jet is modified by Doppler boosting and light travel time effects that depend on the observer's orientation. We can readily show that this geometry can account for a large bending and consequent misalignment between fast-moving VLBI features and any extended structure.

Let the observer's line of sight make an angle Ψ with the cone axis. The jet will be observed to be brightest when it makes an angle $\leq \gamma^{-1}$ with the line of sight. If $\phi \gg \gamma^{-1}$, this implies that for the strongest sources, the observer direction must lie in the hollow cone, $\phi + \gamma^{-1} \geq \Psi \geq \phi - \gamma^{-1}$. Assuming that the emissivity of the jet decreases away from the apex, the source will again appear brightest when the central region points within an angle γ^{-1} at the observer direction, which it will do for a fraction $\sim (2\pi\gamma\phi)^{-1}$ of each precession period. When observed from this direction, a precessing jet will be brightest and appear to bend through an angle ~ 1 radian. Individual inhomogeneities will, however, appear to move rectilinearly. Superluminal expansion of inhomogeneities convected outwards by the jet with apparent

speed $\sim \gamma c$ will occur. Curvature of this order has been reported in a number of superluminal radio sources². For 3C273 and 3C345, continuous bending through an angle $\sim 30^\circ$ is visible on angular scales θ from ~ 5 to ~ 30 m arc s. If the distance of the source is d , we can infer the necessary precession period using the fact that the projected expansion speed is $\sim \gamma c$ to obtain,

$$P_{\text{prec}} \sim 2\pi\phi\theta d/c \quad (9)$$

For 3C273, we substitute $\phi \sim 60^\circ$, $\theta \sim 30$ m arc s, $d = 800$ Mpc to obtain $P_{\text{prec}} \sim 500$ yr. We could obtain this short precessional period if $r_{16} \sim 1.7$, $M_8 \sim m_8 \sim 1$, $t_{\text{GR}} \sim 3 \times 10^6$ yr. More distant sources require somewhat larger and more probable precessional periods. If, as we have assumed, $\phi \gg \gamma^{-1}$, then at large distance from the nucleus the jet will not be subject to large Doppler shifts.

Orbital motion at the source of a jet is another possible source of jet curvature¹⁸. However, the maximum transverse displacement in a single wavelength is of order the binary separation r , far too small to account for observed bent superluminal sources, though possibly relevant to small scale bends in sources like NGC6251 (ref. 18) which has a jet 1 pc long, misaligned by 3° . This would be expected from a binary with separation $r \sim 10^{17}$ cm.

We have argued that there are good reasons to expect binary black holes in galactic nuclei and that most observed examples will have separations in the range 10^{16} cm $\leq r \leq 10^{19}$ cm. Wide binaries may be responsible for inversion-symmetric features in large scale jets. Very close binaries may produce bending and misalignment in VLBI jets through the kinematic consequences of precession. Orbital motion may be reflected in the profiles of permitted emission lines; short orbital periods (~ 10 yr) may even be detectable photometrically using archival plate libraries.

If the binary hypothesis is correct, then we expect that jets that appear to be orbiting or precessing should be associated with cD or irregular galaxies in clusters or groups with large numbers of close companions. Furthermore, estimating the rate of merging and making an evolutionary correction we can compute the frequency of black hole coalescence events from objects at large redshifts to be in the range 10^{-3} – 1 yr⁻¹ (refs. 4, 5). These events (hypernovae) would be accompanied by detectable bursts of electromagnetic and gravitational radiation of powers that can, in principle, approach $c^5 G^{-1} \sim 10^{59}$ erg s⁻¹ for a duration of ~ 1 h. However, as with a supernova, the actual peak electromagnetic power will be moderated by the amount of gas surrounding the explosions, and the strength of the gravitational radiation pulse by a fairly small efficiency factor⁵.

Of course the evolutionary properties of binaries could be far richer than we have described. For example, drawing an analogy with stellar evolution, if one of the holes possessed a massive extended disk or envelope in which the inflow time is as long as $|r/\dot{r}|$ then mass transfer between the components could be important and may even account for much of the variability associated with quasars. Also, merging may occur more rapidly than evolution of the binary and three or more black holes may collect in the core. In this case a gravitational sling-shot¹⁹ rather than coalescence will probably occur.

The detection of a stellar cusp around a central mass, as has been claimed for M 87 (ref. 20), implies that this mass is probably not a recently merged binary, because if black holes were brought together by dynamical friction they would have scoured out all the stars passing within r_h and there should actually be a deficit of stars in this region. The cusp may, however, be restored if a large mass of gas has subsequently been accreted thus repopulating the cusp.

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Spectral broadening measurements of the ionospheres of Jupiter and Saturn

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When Pioneers 10 and 11 flew by Jupiter, their 2.3-GHz monochromatic radio signals underwent substantial spectral broadening as they propagated through the planet's ionosphere^{1,2}. Although spectral broadening has been observed during radio occultation by the solar corona^{3,4}, the Pioneer measurements of Jupiter represent the first time it has been observed during radio occultation by a planetary atmosphere. The Pioneer data are also unique in that r.f. spectral broadening, a scattering phenomenon normally associated with strong intensity scintillations ($\sigma_x^2 \sim 1$ where σ_x^2 is the variance of the log-amplitude scintillations), occurred when the intensity scintillations were weak. This unusual opportunity makes it possible to compare the theory for spectral broadening with the more widely used weak intensity scintillation theory^{5,6}. We report here that good agreement is found, and present the inferred characteristics of the electron density irregularities for the ionospheres of both Jupiter and Saturn. The Saturn results are based on measurements made during the 1979 Pioneer 11 encounter of Saturn.

The details of the Pioneer 10 radio occultation experiment have been discussed elsewhere^{1,2,7}. Figure 1 shows the measured r.f. spectrogram at 2.3 GHz for the lower ionosphere (~ 200 – 500 km altitude region designated region C by Woo and Yang⁷, where altitude is determined by reference² to the atmospheric level at which the neutral gas has a refractivity of 10), measured during exit occultation which took place at 26° N latitude when the solar zenith angle was about 81° . The electron density irregularities in this region have been found to be isotropic⁷. The inherent linewidth over a comparable integration time as determined from measurements outside the ionosphere is less than 0.5 Hz, and can therefore be ignored when interpreting Fig. 1. The three-dimensional spatial wave number spectrum of the refractive index fluctuations corresponding to a power-law spectrum can be written as^{6,7}

$$\Phi(x, \kappa) = \frac{\Gamma(p-1)}{4\pi^2} \sin\left[\frac{\pi(p-3)}{2}\right] c_{no}^2 \exp\left[\left(\frac{x-L}{a}\right)^2\right] \kappa^{-p} \quad (1)$$

where Γ is the gamma function, κ is the spatial wave number, p is the spectral index, c_{no} is the structure constant of refractive index fluctuations, x is the wave propagation direction, L is the spacecraft to planetary limb distance, and a is the extent of the region of irregularities in the x direction.

Figure 2 shows a plot of the structure wave function $D(\tau)$ divided by 2 (refs 4–6). For the power-law density spectrum, a log-log plot of $D(\tau)$ against τ gives an estimate of the spectral index directly from the slope of a linear fit to the data. We have

drawn the dashed line which corresponds to $p = 3.3$, and it can be seen that it represents a very good fit to the data. The power spectrum $W_x(f)$ of the log-amplitude scintillations over the same time interval has been studied previously⁷ and is reproduced in Fig. 3 with the theoretical fit corresponding to $p = 3.3$. The relevant parameters for the theoretical fit are $v = 3.5 \times 10^4 \text{ m s}^{-1}$ (v is the transverse speed of the line-of-sight path), $a = 1.5 \times 10^7 \text{ m}$ and $L = 2.9 \times 10^8 \text{ m}$. It is clear that the intensity scintillation and spectral broadening measurements are consistent in showing that the density spectrum is power-law with spectral index about 3.3. Comparison of the structure wave function with W_x shows that the former is a more sensitive estimator of p . Thus, we conclude that p is 3.3 and not 11/3 (Kolmogorov) as obtained from earlier studies of intensity scintillations alone⁷.

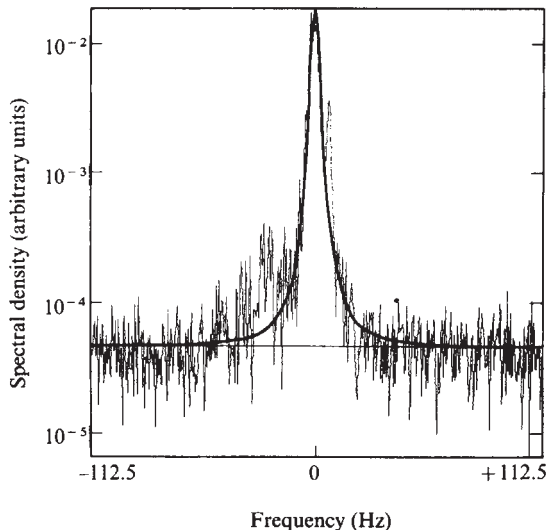


Fig. 1 R.f. spectrogram of Pioneer 10 2.3-GHz signal during Jupiter exit occultation (region C, see text). The heavy solid line is the $p = 3.3$ model discussed in the text, added to an estimate of the system noise (light horizontal line)

For the density spectrum given by equation (1), the variance σ_x^2 of the log-amplitude scintillations can be obtained⁸

$$\sigma_x^2 = \frac{\Gamma(p-1)}{4} \sin \left[\frac{\pi(p-3)}{2} \right] c_{no}^2 \sqrt{\pi} a \Gamma \left(\frac{2-p}{2} \right) \cos \left[\left(\frac{2-p}{4} \right) \pi \right] k^{(2-p)/2} L^{(p-2)/2} \quad (2)$$

where k is the free space wave number and equation (2) is valid whenever $\sigma_x^2 < 1$. The bandwidth B , which represents the full width between frequencies enclosing half the area under the r.f. spectrum, is related to σ_x^2 by⁸

$$B = \frac{Av}{2\pi} \sqrt{\frac{k}{L}} \left\{ \frac{4\sigma_x^2}{\cos \left[\left(\frac{2-p}{4} \right) \pi \right] \Gamma \left(\frac{p}{2} \right) 2^{p-1}} \right\}^{1/(p-2)} \quad (3)$$

where A is a constant having a value of about 1.95 for $4 \geq p \geq 3$. Using the measured noise-corrected σ_x^2 of 0.27 and $p = 3.3$, equation (3) gives $B = 2.5 \text{ Hz}$. We have superimposed on Fig. 1 the theoretical shape of the spectrum corresponding to these values. As can be seen, the agreement is very good.

It is significant that the spectral broadening and intensity scintillation measurements agree so well because this is the first time such a comparison could be made in weak scintillation conditions. When the scintillations become strong, as may be the case when L is large, intensity scintillations become difficult to interpret while the theory for spectral broadening remains valid^{5,6}. In this case, spectral broadening becomes the more useful radio observation for measuring the density spectrum. In

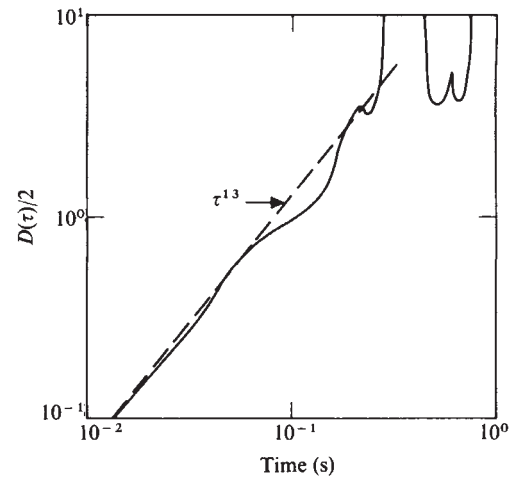


Fig. 2 Structure wave function divided by 2 for spectrum of Fig. 1. The linear fit (dashed line) corresponds to the $p = 3.3$ model.

equation (1) we have assumed that the irregularities are isotropic as is the case for the region C data discussed here. In the upper ionosphere the irregularities are anisotropic because of alignment with the planetary magnetic field⁷. Theoretical results for interpreting spectral broadening measurements taking into account anisotropic irregularities and a general power-law density spectrum are available⁹.

Note from equations (2) and (3) that although $\sigma_x^2 \sim L^{(p-2)/2}$, B is independent of L but is proportional to v . Thus, like multipath propagation², intensity scintillations become more severe when the distance between the planet and spacecraft is increased (as long as $\sigma_x^2 \leq 1$). On the other hand, the amount of spectral broadening is unchanged as long as v is unchanged. It is also clear from equation (3) that the substantial spectral broadening observed by Pioneers 10 and 11 was due to the very large fly-by velocities, rather than strong intensity scintillations.

The one-dimensional spectrum of the electron density fluctuations is of particular interest because it corresponds to the spectrum measured directly by spacecraft. For the case discussed above, we have $V_{ne}(\kappa) = 2.1 \times 10^{26} k^4 c_{no}^2 \kappa^{-1.3}$. Applying equations (2) and (3) to the results shown in Figs 1–3, we obtain $c_{no} = 8.3 \times 10^{-10} \text{ m}^{-0.15}$ so that $V_{ne}(\kappa) = 7.8 \times 10^{14} \kappa^{-1.3} (\text{el/m}^3)^2 / \text{m}^{-1}$ for $5 \times 10^{-4} \leq \kappa \leq 2 \times 10^{-3} \text{ m}^{-1}$.

A radio occultation experiment of Saturn was carried out during the recent Pioneer 11 fly-by¹⁰. We have studied the intensity scintillation and spectral broadening measurements

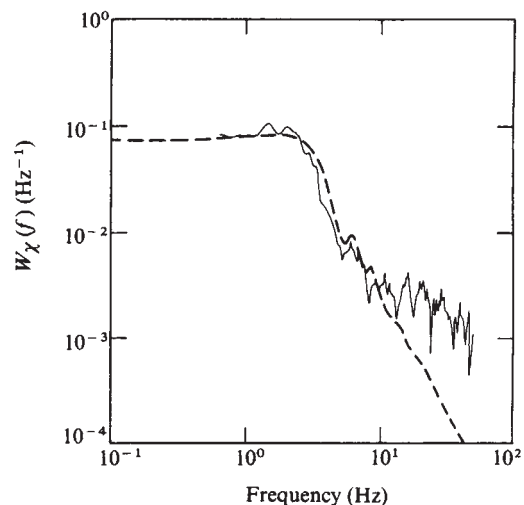


Fig. 3 Fluctuation power spectrum of the log-amplitude scintillations for region C data. Smooth dashed curve is the $p = 3.3$ model. System noise level is about $2.5 \times 10^{-3} \text{ Hz}^{-1}$; the roll-off at high frequencies is due to digital filtering.

made by the ionosphere during exit occultation at latitude 9.5°S and solar zenith angle 90.6° . Within the instrumental limits of the radio system, neither intensity scintillations nor spectral broadening was detected. Using the relevant parameters for the Saturn occultation ($v = 2.44 \times 10^4 \text{ m s}^{-1}$, $L = 1.2 \times 10^8 \text{ m}$ and $\alpha = 1.6 \times 10^7 \text{ m}$) and assuming a density spectrum with $p = 3.3$, we deduce that $c_{\text{no}} \leq 3.4 \times 10^{-10} \text{ m}^{-0.15}$. The corresponding one-dimensional spectrum for the electron density fluctuations is then $V_{\text{ne}}(\kappa) \leq 1.3 \times 10^{14} \kappa^{-1.3}$ where $\kappa \geq 7.8 \times 10^{-4} \text{ m}^{-1}$. The Pioneer 11 measurements, therefore, show that the electron density fluctuations are smaller in the ionosphere of Jupiter. This may not be too surprising because the mean electron densities are higher in the ionosphere of Jupiter² than in Saturn¹⁰. Note that the Pioneer measurements of Jupiter and Saturn represent the observations of only a few locations and that globally these irregularities may vary substantially, as in the case of the Earth's ionosphere¹¹.

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Galileo's observations of Neptune

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The planet Neptune was discovered in 1846. As its period of revolution is almost 165 years, Neptune has not yet completed one revolution since its discovery. Largely as a result of this, its orbit is not known with a precision comparable to that of the inner planets. A pre-discovery measurement of Neptune's position by Lalande in 1795 differs from the predicted position by 7 arc s. There is some debate about whether this discrepancy is real or an error of measurement¹. Clearly, it would be worthwhile to find other pre-discovery observations of this planet. One possible way of finding such observations is to search for close approaches of Neptune to other objects which were frequently observed. Neptune was actually occulted by Jupiter in January 1613 and September 1702 (ref. 2). By 1702 the telescope was in widespread use, and examination of manuscripts of that period should reveal cases where Neptune was seen near Jupiter and mistaken for a star. The abundance of possible material, however, makes a search for such observations lengthy. We have found that Galileo observed the planet Neptune on 28 December 1612 and 28 January 1613. The latter observation may be of astrometric value, and differs by 1 arc min from the predicted position of Neptune. Galileo also detected the motion of Neptune.

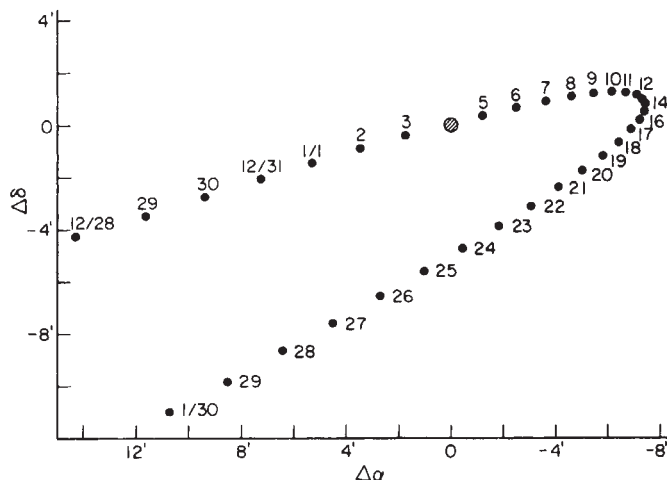


Fig. 1 The positions of Neptune with respect to Jupiter, 28 December 1612 to 30 January 1613. Black dots, Neptune; cross-hatched circle, Jupiter.

We began by examining Galileo's observations of 1612–13. Galileo observed Jupiter almost nightly at that time, and sometimes more than once on the same night. His notebooks have been published in several editions.

Figure 1 shows the ephemeris positions of Neptune with respect to Jupiter for 28 December 1612 to 30 January 1613. The field of view of Galileo's telescope was about 15 arc min, so both planets were within the same field for the entire interval. Jupiter became retrograde in mid-January, and so passed Neptune twice.

Figure 2 shows portions of a page of Galileo's notebooks which cover some of this time interval. In these drawings of Jupiter and its satellites, north is to the upper right, and east to the upper left. The first observation is dated: "1612 December, day 27, hour 15:46 a.m.". This corresponds to 28 December, 0346 a.m., local time. (Galileo considered each day to begin at noon.) A 'fixed star' is marked to the east of Jupiter on this date. The Palomar Sky Survey prints show no bright star in this position. There are, in fact, very few bright stars in that entire region of the sky in the constellation of Virgo. (Galileo was occasionally able to see stars of magnitude 9, but none fainter. Neptune is always brighter than magnitude 8. The galilean satellites have magnitudes of 5 to 6.) We have found that the direction of Galileo's 'star' from Jupiter is almost exactly the direction of Neptune on that night (Fig. 3). It would not have been possible to draw the distance of Neptune to scale, since the planet would have been off the edge of the page, so he put it at the edge of the page and indicated its direction with a dotted line.

Table 1 Mean positions of the objects for 28 January 1613, 2300 UT

Jupiter (from JPL DE 102)	$\alpha = 12 \text{ h } 04 \text{ min } 59.54 \text{ s}$ $\delta = +1^\circ 02' 11.0''$	1950.0
SAO 119234 (includes proper motion)	$\alpha = 12 \text{ h } 05 \text{ min } 27.02 \text{ s}$ $\delta = +0^\circ 54' 10.8''$	
Neptune (from JPL DE 102)	$\alpha = 12 \text{ h } 05 \text{ min } 33.3 \text{ s}$ $\delta = +0^\circ 52' 24''$	1950.0
Galileo's position of Neptune	$\alpha = 12 \text{ h } 05 \text{ min } 30.5 \text{ s}$ $\delta = +0^\circ 53' 11''$	

Galileo's position of Neptune with respect to the star is probably accurate to better than 10 arc s as judged from his accuracy in measuring the positions of the satellites. The accuracy of the position of the star itself depends on the accuracy of its proper motion, as listed in the SAO Star Catalog. We have used a total proper motion of 0.13 s in RA, and 11.5" in declination.

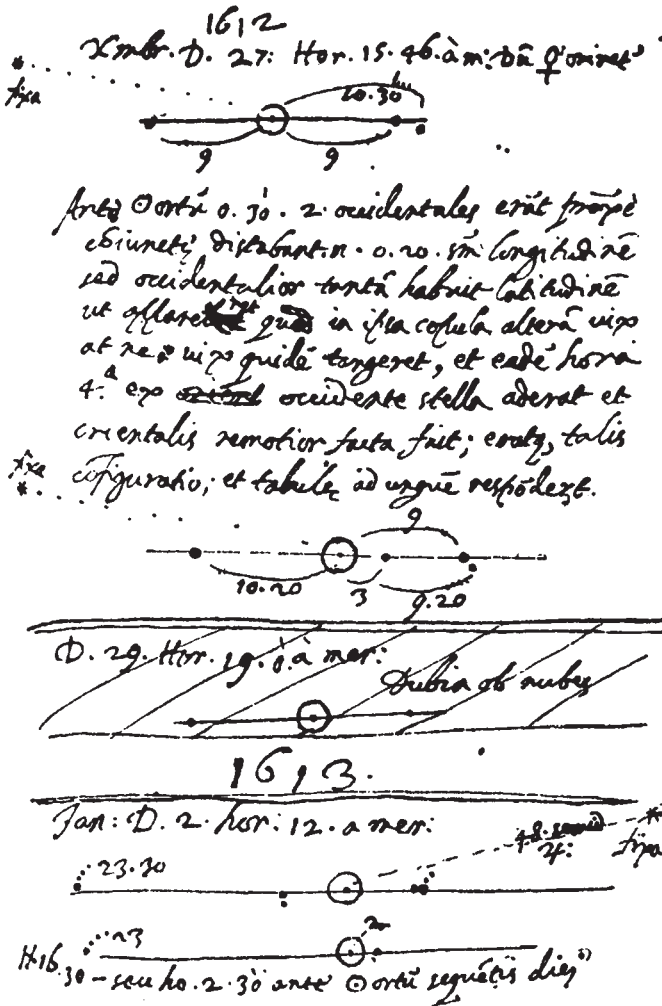


Fig. 2 Portions of a page from Galileo's notebooks, for 27/28 December 1612 to 2/3 January 1613 (ref. 3).

Figure 3 shows the ephemeris positions of the jovian satellites and Neptune for 0300 UT, 28 December 1612, together with the indicated position of Galileo's 'fixed star'. The exact correspondence of the directions and the absence of any bright stars in that area make us confident that Galileo actually saw Neptune on this night—234 yr before that planet was discovered.

Returning to Fig. 2, we see a star marked "48 jovian radii" to the north-west of Jupiter on "1613 January, day 2, hour 12 midnight". This star was easily identified as SAO 119234, visual magnitude 7.1. The star was actually 52 radii from Jupiter at the time. Again, this star would have been off the edge of his notebook paper, so Galileo drew it at the edge of the page.

Galileo must have seen Neptune during each of the following nights. It is puzzling that he did not show the planet in his drawings. Perhaps he did not want to clutter his drawings of the satellites with 'extraneous' objects near the satellite plane.

Figure 4 shows two observations from the end of January 1613. On "day 27" we again find SAO 119234, 20 jovian radii south-east of Jupiter. On the following night, two stars are shown in a separate drawing. The time of this latter observation is given as "day 28, 6 hours after sunset". This corresponds to about 2300 local time on 28 January. A plot of the ephemeris positions of Jupiter, Neptune and the star (Fig. 5), shows immediately that "star 'a'" is SAO 119234, while "star 'b'" is definitely Neptune. Galileo's note in the lower left quarter of this drawing may be translated as follows:

Beyond fixed star "a", another followed in the same straight line, this is "b", which was also observed on the preceding night, but they (then) seemed farther apart from one another.

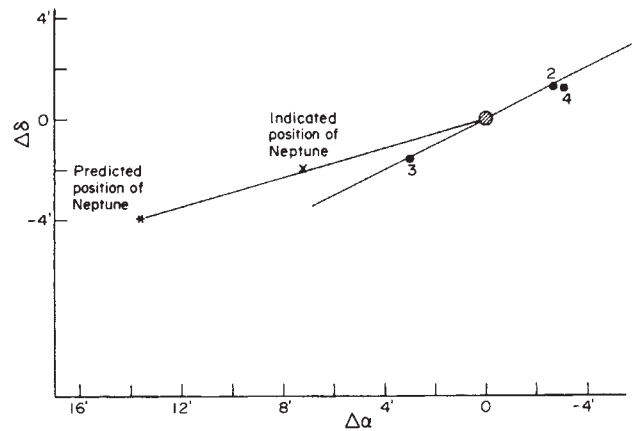


Fig. 3 The positions of Neptune and the jovian satellites, with respect to Jupiter, for 28 December 1612, 0300 UT. All of the positions in this paper are from Development Ephemeris 102, Jet Propulsion Laboratory, Pasadena, California.

Mss. Gal., P. III, T. IV, car. 140r.

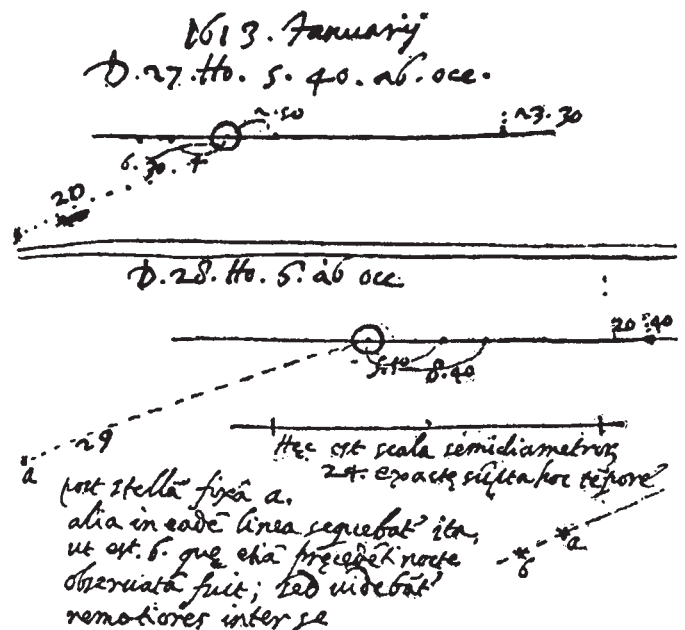


Fig. 4 Galileo's notebook for 26/27 and 27/28 January 1613.

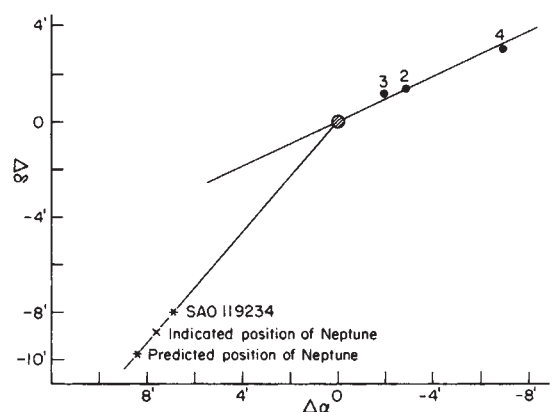


Fig. 5 The positions of Neptune, the jovian satellites and a star, for 28 January 1613, 2300 UT.

In other words, not only did Galileo see Neptune, he also noticed that it had moved with respect to a fixed star. Without an adequate telescope mounting it would have been impossible for him to follow up this observation after Jupiter had moved on, even if he had thought of doing so.

This observation of Neptune may be of astroinetric value. Immediately below the drawing of Jupiter and its satellites is a linear scale showing 24 jovian radii. Below this scale is a drawing of the two 'stars'. The drawing indicates that Neptune was 3.75 radii, or just over 1 arc min, from star 'a'. The ephemeris shows that Neptune should have been 2 arc min from the star. It is therefore possible that the ephemeris is incorrect by about 1 arc min—a relatively large amount in celestial terms (see Table 1). We see no reason to doubt that Galileo drew the stars to the indicated scale, although it is surprising that the 'incorrect' ephemeris position still lies on the same straight line from Jupiter and the star. In principle, the error in the ephemeris could be in any arbitrary direction, most likely along the ecliptic, which is not parallel to the line from Jupiter.

We are left, then, with the strong possibility that the ephemeris of Neptune is in error by a significant amount, which would require a revision of the orbital elements of that planet, and suggests the existence of an unknown perturbation. [One of us (S.D.) believes that such revisions may disclose an occasion on which Galileo mistook Neptune for a satellite near Jupiter. Eclipse conditions and an ambiguity in timing one of his observations suggest this as a possibility, even if remote.]

The ephemeris of Neptune used in this work is from Development Ephemeris no. 102 of the Jet Propulsion Laboratory. Other ephemerides will give different results. We hope that this work will help decide which ephemeris is most accurate. It would be most interesting to try to fit the Galileo position and the Lalande positions into a new ephemeris of Neptune.

We thank Drs Jay Lieske and E. Miles Standish for supplying ephemerides of Jupiter, Neptune and the galilean satellites which made this work possible, and Dr Alexander Pogo for help and advice. C.T.K. is supported by grants from the National Aeronautics and Space Administration, and the NSF.

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Imaging and focusing of neutrons by a zone plate

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The use of refractive lenses for long-wavelength neutrons, although possible¹, is severely limited by the fact that the refractive index differs only very slightly from unity (for example, $n - 1 = 2.3 \times 10^{-4}$ for quartz at $\lambda = 20 \text{ \AA}$). However, the fact that only a relatively thin layer of material is needed to produce an optical path difference of half a wavelength (for example, $2.4 \text{ \mu m}$ of Cu at $\lambda = 20 \text{ \AA}$) led us to propose² previously that neutrons could be focused by diffraction from a phase grating of variable spacing; this is, in fact, a type of zone plate. Zone plates, first described by Soret³, have been used satisfactorily over a large part of the electromagnetic spectrum^{4–6}. We now report the successful focusing and imaging of a slow neutron beam by means of a Fresnel zone plate⁶.

Conventional zone plates work by blocking out alternate zones. Constructive interference of the waves diffracted from the remaining zones occurs at specific points which are in an

image relationship to their original source. Lord Rayleigh⁷ pointed out that phase reversing, rather than absorbing alternate zones, would achieve a focus with greatly enhanced intensity. In the absorbing zone plate, only half the incident amplitude (leading to one-quarter the intensity) is focused; in addition, a 0 order beam of unfocused waves is present. This is not the case with the phase reversal zone plate in which the 0 order is absent and much more of the energy is focused. These facts were experimentally verified by Wood⁸.

The geometric pattern of a Fresnel zone plate (see Fig. 1) is precisely that of the interference pattern of two spherical waves of differing radii of curvature. Thus, the zone plate may be regarded as the hologram of a point. This fact suggested our first approach to the construction of zone plates for long wavelength neutrons, along the lines previously used by others⁶ in producing zone plates for soft X rays. However, we subsequently found that higher quality zone plates could be produced by the successive photographic reduction of a computer drawn pattern.

Our zone plates, which are of the phase reversal type, were produced in Melbourne by photolithographic techniques close to the limits of resolution used by the microelectronics industry. A thin layer of positive photoresist (Shipley AZ 1350 J) on a silicon wafer substrate was exposed to UV light through a mask carrying the zone plate pattern. After dissolving away the unexposed photopolymer, a layer of copper was electrolytically deposited on to the exposed surface. (Before coating with photoresist, a thin layer of chromium was deposited on the substrate to enhance the adhesion of the copper.) The thickness of the copper deposited was strictly controlled to be equal to $D(\lambda/2) = 2.4 \text{ \mu m}$, the thickness calculated to give a phase reversal to neutrons of $20 \text{ \AA}$ wavelength.

$$D(\lambda/2) = \frac{1}{2}\lambda/(n-1) \quad (1)$$

where

$$n = 1 - \lambda^2 Nb/2\pi \quad (2)$$

for a material having N scattering centres per unit volume, each with scattering length b .

The final zone plates produced in this way had the following characteristics: diameter $d = 2 \text{ mm}$, number of zones $\xi = 200$, width of outermost zone $\delta = 2.5 \text{ \mu m}$, focal length $f \text{ (m)} = 50/\lambda \text{ (\AA)} = 2.5 \text{ m}$ at $\lambda = 20 \text{ \AA}$. Note that the width, δ , of the outermost zone, limited by the resolution of our plating technique, determines the quality of the lens in the sense that its F -number (f/d ratio) may be shown to be given by

$$F = \delta/\lambda \quad (3)$$

Thus, our lens has $F = 1,250$, which is poor compared with lenses used with visible light but comparable with the performance of lenses used in electron optics. The reasons for this are most easily understood by reference to the holographic aspects of the problem. Regarding the zone plate as a hologram of a point, recorded with light of a given wavelength but reconstructed with waves of wavelength 250 times shorter, it is

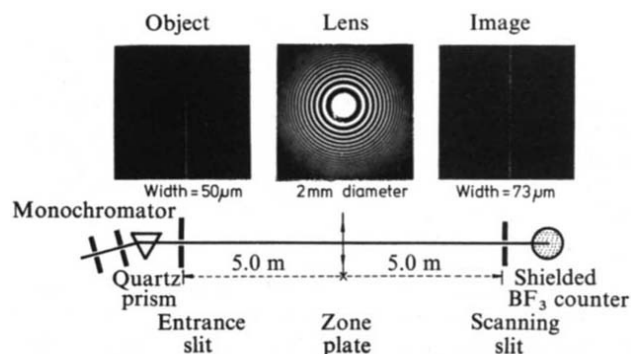


Fig. 1 Schematic layout of neutron focusing experiment.

easily shown that the ultimate resolution is still that attainable with visible light. Therefore, better quality zone plate lenses can only be produced by methods avoiding the use of visible light, such as electron beam lithography.

We tested our zone plate on the long wavelength neutron beam, H18, at the High Flux Research Reactor of the Institut Laue-Langevin in Grenoble. The main features of the apparatus are shown in Fig. 1. The monochromator, consisting of a 120° quartz prism with entrance and exit slits, was used to select a 1-Å wide wavelength spread centred on 20 Å. The monochromator exit slit, set to 50 µm width, served as the object to be imaged by the zone plate at 1:1 magnification, that is, the object and image distances were both 5 m. The image was scanned in 20-µm steps by a 30-µm wide slit, placed in front of a shielded BF₃ proportional counter. A Polaroid camera with a lithium-loaded ZnS scintillator screen was also used to photograph the beam in the object and image positions, giving the results shown in Fig. 1.

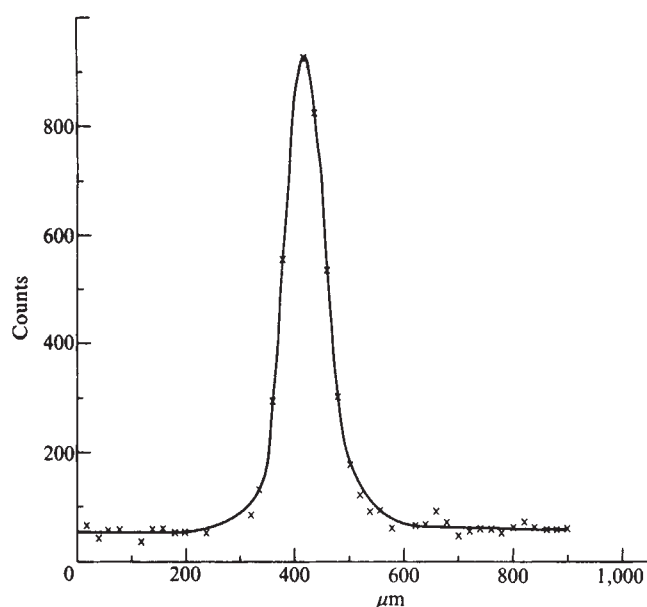


Fig. 2 Neutron intensity in the image plane.

Measurements of the beam detected in the image plane are shown in Fig. 2, and gave FWHM = 73 µm, a value consistent with the instrumental resolution, combined with the calculated chromatic aberration. Because object and image distances were fixed by the lengths of vacuum tanks taking up the flight paths, longitudinal scanning of the focus was not possible with this apparatus. We did, however, verify the calculated focal length by observing the width of the image as the wavelength was varied by rotating the monochromator prism. The image width showed a sharp minimum at 20 Å. An estimate of focusing efficiency (the fraction of neutrons going through the lens aperture that are focused into the image) was obtained by comparing counting rates at the image position with and without the lens. This gave 40 ± 10%, in agreement with theoretical expectations.

The applicability of the zone plate lenses described above is limited by the smallness of their aperture, their inherent chromatic aberrations and the low intensities of available long wavelength neutron beams. Nevertheless, there are several interesting possibilities in the field of neutron optics, some of which we have described previously².

We thank the directors and staff of the Institut Laue-Langevin for their hospitality and encouragement, the University of Melbourne for travel grants, Professor J. Kalus of the University of Bayreuth for allowing us the use of his apparatus, R. L. Shaw and P. Francis for their help with the production of holographic

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Electron irradiation induced vitrification at dislocations in quartz

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Recent observations using the 'weak beam' electron microscopy technique of split images of dislocations in quartz have been interpreted as arising either from dissociation of the dislocations into partials¹, or from a glassy (metamict) phase at the core², resulting from irradiation damage. In this letter we describe observations of lattice images of near end-on dislocations in quartz, which enable us to distinguish between these two models.

Quartz, the second most abundant crustal mineral, is important in the study of deformation within the Earth's crust. Plastically deformed quartz has therefore been the subject of numerous studies³⁻⁵ from which two significant findings have emerged: (1) quartz deforms by dislocation creep mechanisms within the deeper crust and (2), trace amounts of lattice-bound OH cause a marked softening of quartz ('water weakening'). The causes of water weakening are not known with certainty but it is thought to arise from increased glide and/or climb velocities⁶⁻⁹. Any detailed model for this effect and for the interpretation of stress-strain curves requires a knowledge of the structures of the dislocation cores in quartz. Two different models have been proposed for the commonly encountered α -dislocations: Trepied and Doukhan¹ observed a splitting of weak beam images during irradiation in the microscope which they attributed to the movement of partial dislocations bounding a stacking fault composed of a complex twin structure; Cherns *et al.*² re-examined the image splitting and proposed an alternative model whereby irradiation damage led to the preferential formation of a metamict region in the vicinity of the dislocation core. The observed widening of the split weak beam images from about 6 nm to several tens of nm was then attributed to additional radial displacements around the dislocation, generated by the expansion due to the crystalline-amorphous transition.

We have now attempted high-resolution lattice imaging of deformed quartz to obtain further information about core structures of essentially unirradiated dislocations, and to investigate in detail the development of any metamict phase. We used the naturally deformed quartz from the Cap de Creus shear zones in north-east Spain¹⁰; detailed textural and electron microscopic studies had been completed on this material, and

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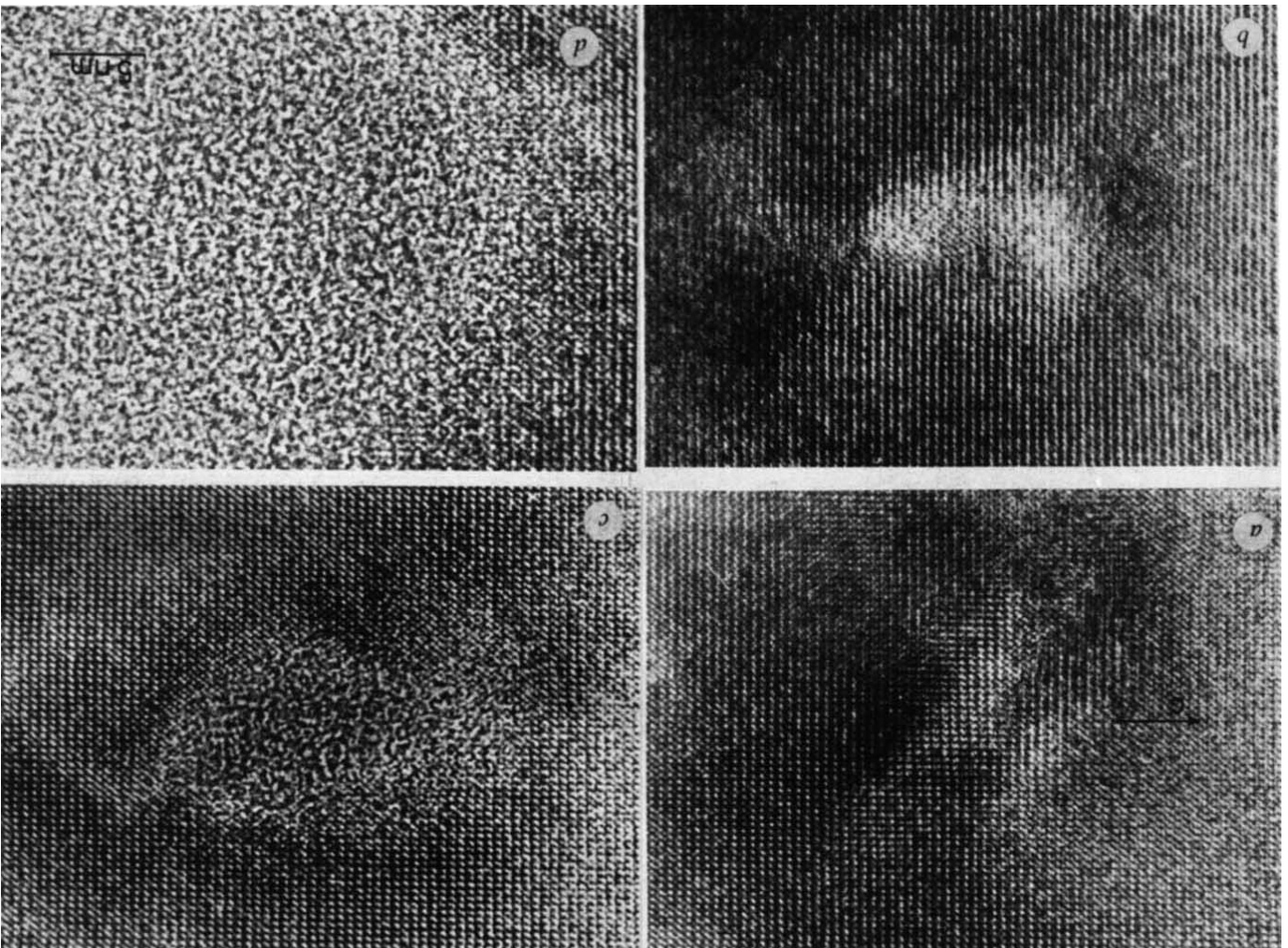


Fig. 1 *a*, (1120) lattice image of a defective region in quartz, before electron beam damage becomes visible. *b*, The same region of crystal, after a further 10 s of exposure to the electron beam. *c*, After another 10 s in the beam a region of 'amorphous' contrast has appeared in the core region. *d*, After a total irradiation time of about 40 s most of the field of view has been replaced by the 'glassy phase'. Magnification, $3 \times 10^5 \times$.

not possible because the dislocation geometry and diffracting conditions are not sufficiently well defined. A slightly later stage of the irradiation is shown in Fig. 1*a*. The strong diffraction contrast at the dislocation has almost disappeared, to be replaced by a lighter region diffracting less strongly than the surrounding area. Within this region the lattice image may still be discerned, but less clearly. In Fig. 1*c*, a central oval-shaped region has developed, displaying 'amorphous' contrast typical of a glass, whereas the regular lattice image is still clearly visible in surrounding regions. In Fig. 1*d* most of the field of view appears amorphous. The contrast between Fig. 1*c* and 1*d* is clearly indicative of a transition from crystalline to metamict quartz, and strongly supports the model proposed by Cherns *et al.*². Vitrification of the dislocation core due to electron irradiation has also been suggested previously by Ardell *et al.*¹¹. The [1100] directions, and is oval with its long axis in the *c* direction. While the images show no evidence of any pronounced dislocation into partial dislocations on the basal plane, as envisaged by Trepiéd and Doukhan¹, to be accompanied on their model by displacements along [1100] in the [1120] projection, the possibility of dissociation over a width of less than 1 nm cannot be ruled out. The absence in Fig. 1*a* of evidence for an amorphous region suggests that the image splitting of about 6 nm reported by Cherns *et al.*² in the first micrograph of their irradiation sequence is due to the irradiation induced metamict phase. However, we cannot exclude the possibility of the presence of an amorphous region a few tenths of a nanometre in

consequently it was known to contain *a*-type dislocations lying in the basal (0001) plane. Samples were cut normal to the basal plane and within a few degrees of a [1120] pole. Dislocations of two of the three *a*-types, with Burgers vectors $\mathbf{b} = \frac{1}{2}\mathbf{a}$ [1210] and $\frac{1}{2}\mathbf{a}$ [2110] and with line orientations close to 60°, preferred in these specimens, are close to end-on in a foil oriented at the [1120] pole, if they exhibit dot contrast in diffraction contrast images. Specimens were ion-thinned and examined in a JEOL JEM 200CX electron microscope operating at 200 kV, with a high-coherence Lab₆ electron source and an objective lens with spherical aberration coefficient of 1.1 mm. A minimal exposure technique was used to reduce beam damage effects, photography being carried out on regions adjacent to those used for orientation, stigmation and focusing adjustments. [1120] lattice images were recorded on Kodak 4463 film at a magnification of $4 \times 10^5 \times$. Figure 1 shows an irradiation sequence of an area containing a nearly end-on dislocation, as judged by the dot contrast criterion at lower magnification. The total irradiation time between Fig. 1*a* and *d* was rather less than 1 min. Figure 1*a* shows the earliest stages of the irradiation. In areas well away from the dislocation core the image reflects the quartz structure projected down [1120]. By construction of a Burgers circuit in the undistorted region the presence of a dislocation was confirmed, the Burgers vector being consistent with one of the expected types. In the vicinity of the dislocation core, near the centre of the micrograph, the regular lattice is distorted; direct interpretation in this region is

diameter ('colloidal cores' as postulated by Tsinzerling¹²). Finally, note that because of the additional displacements generated by the growth of the metamict phase, great care must be taken in the determination of Burgers vectors of dislocations in quartz.

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Silica retards radial growth of spherulites in isotactic polystyrene

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We recently reported the retardation of the rate of crystallization, measured calorimetrically, in isotactic polystyrene (iPS) nucleated with silica (Cab-O-Sil) that had been treated with trimethylsilane¹. The loading of the nucleant was 5 g per 100 g of polymer or two parts per hundred (p.p.h.) by volume, of polymer. From microscopic observations it was known that the number of nuclei present in the nucleated system was at least double that present in the pure polymer in identical cooling conditions². The only rationale available for the retardation effect seemed to be that the nucleant was inhibiting the growth of the crystals and thus the overall rate of crystallization. However, previous workers have reported spherulitic growth rates to be unaffected by the presence of nucleants in systems with loadings up to 3 p.p.h. (refs 3-6). To clarify this apparent paradox the measurement of the radial growth rates of spherulites in the system iPS-silica by photomicrography was undertaken. Here we confirm that silica does retard the rate of growth of such spherulites.

A Zetopan Reichert research microscope equipped with crossed polarizers and a Nikon 35 mm camera were used in conjunction with a Mettler FP52 hot stage (controlled to $\pm 1^\circ\text{C}$ and calibrated using the melting points of benzoic acid, naphthalene and benzophenone). The samples were prepared by melting 0.1-0.2 mg of iPS between a precleaned microscope slide and a coverglass separated by Pt washers $\sim 20\ \mu\text{m}$ thick. Before observation the samples were premelted at 255°C for 5 min. Subsequently, they were cooled to the desired crystallization temperature and the growth rates of spherulites was followed by photographing them at regular intervals. The diameters of 15 and 60 spherulites, respectively, for the un-nucleated and nucleated systems were measured at least five times for each photograph and a growth rate for each spherulite was determined, using a linear least squares program, for diameters in the range 15 to $45\ \mu\text{m}$. An average of the spherulitic growth rates was calculated by taking the average of the growth rates of individual nuclei at a given crystallization temperature.

Figure 1 shows the effect of temperature, in the range 150 – 200°C , on the rate of radial growth of spherulites in un-nucleated iPS (viscosity molecular weight approximately 1×10^6 , equilibrium melting point = 231°C). The growth rates, G , can be described by a modified form of the Lauritzen-Hoffman equation⁷

$$\ln G = \ln G_0 - \left[\frac{C_1}{R(C_2 + T - T_g)} \right] - \frac{4b_0\sigma\sigma_e T_m^0}{k\Delta H T \Delta T} \quad (1)$$

where G_0 is a pre-exponential factor which is independent of temperature. The effect of temperature of the rate of transport in a polymer melt is described by the term $[C_1/R(C_2 + T - T_g)]$ where R is the gas constant, C_1 and C_2 are the WLF constants describing viscous flow in polymers, T is the crystallization temperature, and T_g is the glass transition temperature. The remaining term in equation (1) relates to the nucleation energy which depends on the monomolecular thickness b_0 , the side and end interfacial energies, σ and σ_e , respectively, the equilibrium melting point, T_m^0 , the heat of fusion, ΔH , the Boltzmann constant, k , and ΔT , the supercooling. As shown in Fig. 1, an excellent 'fit' to the growth pattern in the un-nucleated system is obtained when:

$$C_1 = 1.72 \times 10^4 \text{ J mol}^{-1}$$

$$C_2 = 75 \text{ K}$$

$$T_g = 365 \text{ K}$$

and

$$\frac{4b_0\sigma\sigma_e T_m^0}{k} = 9.25 \times 10^4 \text{ K}^2 \quad (T_m^0 = 503.8 \text{ K})$$

The addition of 0.8 vol. % fine particle silica (Cab-O-Sil M-5) caused a decrease in the radial growth rate over the range 170 to 190°C (Fig. 1). To obtain a fit to the data for this system, equation (1) was modified to include a contribution due to the adsorption of polymer segments onto the silica with a heat of adsorption (ΔH_{ads}) known⁸ to be $2.05 \times 10^4 \text{ J mol}^{-1}$, to

$$\ln G = V_2 \ln G_0 - \frac{C_1 + \phi \Delta H_{\text{ads}}}{C'_2 + T - T_g} - \frac{4b_0\sigma\sigma_e T_m^0}{k\Delta H T \Delta T} \quad (2)$$

where V_2 is the volume fraction of polymer and ϕ is a constant. Again, an excellent fit is obtained to the data when

$$\phi = 8.27 \times 10^{-2}, \quad C'_2 = 107 \text{ K}$$

The observed increase in C_2 to C'_2 , that is 75 to 107 K , is in accordance with, but somewhat larger than, directly measured values for the change in T_g in the presence of added silica⁹.

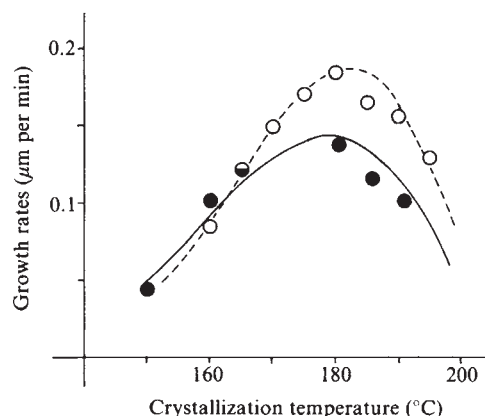


Fig. 1 Effect of crystallization temperature on the spherulite growth rates in un-nucleated (○) and nucleated (●) isotactic polystyrene [--- theoretical, equation (1); — theoretical, equation (2)].

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Interestingly, as would be expected, a satisfactory account for the retardation in the rate of radial growth in iPS due to added silica can be achieved by considering only the transport term in equation (1).

These results demonstrate a decrease in the rate of radial growth of spherulites in nucleated polymer melts. This effect is probably general but has not been previously recognized because, despite the retardation in the rate of development of

the spherulites, the overall crystallization rate will be retarded only in cases where the increase in the number of nuclei is small. The transport limitation has been demonstrated to result from the formation of quasi-crosslinks in the polymer melt as a result of the physisorption of the polymer segments on the nucleant¹⁰.

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Simulation of a lipid monolayer using molecular dynamics

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Numerical simulation is often a useful tool for investigating the behaviour of complex systems with many degrees of freedom. Of the two major methods in this field, the Monte Carlo method and the molecular dynamics method, only the first has been applied to realistic models of lipid monolayers¹⁻⁵. The term lipid monolayer is used here to describe a class of systems consisting of chain molecules on a liquid substrate, the characteristic properties of which can be summarized as follows. (1) The constituent molecules are amphipathic, that is they consist of a hydrophilic (polar) head group and one or more hydrophobic hydrocarbon chains. (2) Due to the amphipathic character of the molecules, the head groups are constrained to the plane of the substrate, whereas the tails are directed outwards from this plane. (3) The collective properties of the molecules are determined by their short-range repulsive and long-range attractive interactions and by the steric repulsion of the tails. We now present what we believe to be the first molecular dynamics simulation of a realistic model of a lipid monolayer. The model system, which has all three properties enumerated above, shows a first order phase transition from an ordered fluid-like state to a disordered, gas-like state.

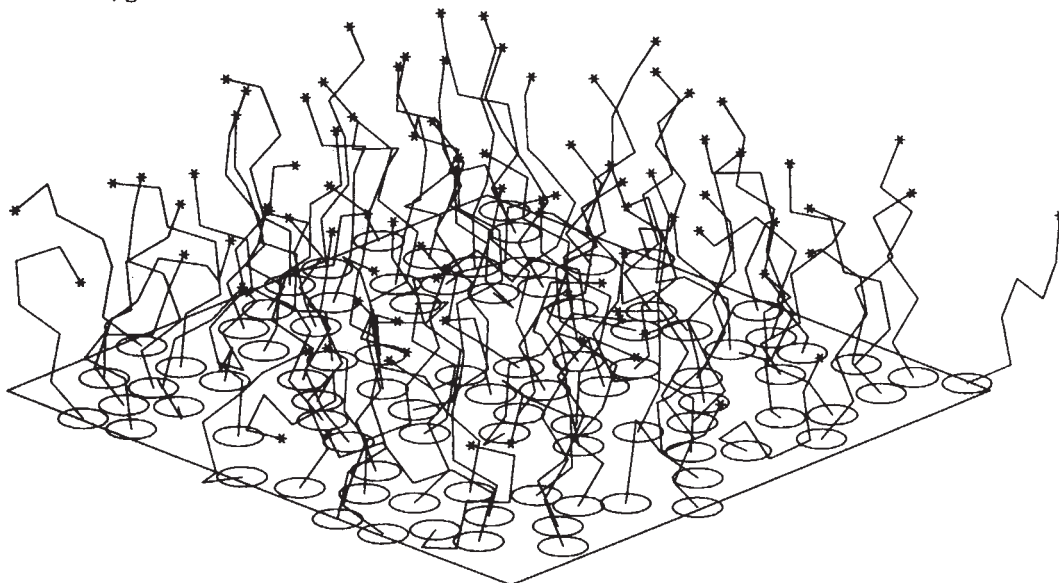
Cotterill⁶ and Toxvaerd⁷ have performed numerical dynamics calculations on very rough models of monolayers. Both authors only consider models in which the individual molecules are reduced to purely two-dimensional objects which move in the plane of the monolayer. The influence of the steric interactions of the chains and of their large number of configurations is therefore neglected. Consequently, these calculations are at best a simulation of the crystalline state of a monolayer, with all lipid chains parallel and in the all-*trans* state. Also, the mechanism believed to be responsible for the main phase transition in lipid monolayers—the ‘melting’ of the tails—is absent in these models. These are rather severe limitations (see ref. 8 for a review of lipid monolayer theory and of some of the terminology used here). To improve this situation we have performed a molecular dynamics simulation of a realistic model of a lipid monolayer. The system consists of 90 chain molecules (lipids), each molecule consisting of seven repeating units (CH₂ groups). The first repeating unit of each chain (the head group) is constrained to move in a plane, thus simulating the interaction between the head groups and the molecules of the substrate. The head groups interact with a 12,6 Lennard-Jones interaction potential with cutoff 2.5σ

$$V(r) = 4 \epsilon \{ (\sigma/r)^{12} - (\sigma/r)^6 \} \quad (r < 2.5 \sigma) \quad (1)$$

$$V(r) = 0 \quad (r > 2.5 \sigma)$$

where r denotes the distance between two head groups, and ϵ and σ are parameters. The geometry of the hydrocarbon chains is simulated in the following way. In each chain, the distance between two neighbouring repeating units is kept fixed, within small fluctuations, by a strong harmonic force, proportional to

Fig. 1 A typical equilibrium configuration of a lipid monolayer for $T^* = 1$ and $\rho^* = 0.4$. T^* is the reduced temperature: $T^* = kT/\epsilon$; ρ^* is the reduced number-density: $\rho^* = \rho/\rho_0$ with ρ_0 the close-packed density. The head group of each lipid is indicated by a circle of radius σ and the end of each lipid by an asterisk. Note the clustering of the head groups and the formation of small pores. These pores, which might play a part in membrane permeability, have also been found previously in an exactly solvable membrane model¹²⁻¹⁴.



$r - \sigma$, which acts to drive the two repeating units back to their equilibrium distance σ . In the same way, a strong harmonic force keeps the distance between any pair of next-nearest neighbours in a chain fixed at the value a . The value of a is chosen in such a way that any two successive C-C bonds in a chain define an angle of 109.5° , in agreement with the experimental value for hydrocarbon chains. With the exception of the head groups, all repeating units in different chains interact with a strong repulsive force proportional to $(\sigma/r)^{20} - 1$, with a cutoff σ . The plane to which the head groups of the lipids are constrained acts as a reflecting plane for all other repeating units, through a strong repulsive force.

Clearly, therefore, our model incorporates most of the features quoted in the first paragraph. However there are three exceptions. (1) The attractive part of the interaction between a pair of chains is concentrated in the head groups instead of being distributed over the whole length of the chains. (2) Although the polar forces between the head groups and the molecules of the substrate have been accounted for, the polar interactions between the head groups themselves are neglected. (3) The energy difference of about 0.35×10^{-13} erg between a *trans* and a *gauche* configuration of a C-C bond has not been taken into account, implying that there is no difference between the liquid expanded and liquid condensed phases.

The properties of this system were determined by numerical integration of the equations of motion of the 630 repeating units, using Verlet's algorithm⁹. The calculations were done on the CDC Cyber 73/28 and CDC Cyber 173/8 of SARA in Amsterdam. We used two-dimensional periodic boundary conditions. The initial configuration, for which the all-*trans* state was chosen, tends to equilibrium after a sufficient number of collisions. We used an adjustment procedure¹⁰ to guide the system to thermodynamic equilibrium at one particular temperature T . A typical equilibrium configuration is depicted in Fig. 1.

Once the system is in thermodynamic equilibrium, the lateral pressure Π can be calculated from the virial theorem¹¹.

$$\frac{\Pi A}{NkT} = 1 - (2N\langle v^2 \rangle)^{-1} \sum_{i \neq j} (\mathbf{r}_{i,j} \cdot \mathbf{a}_{i,j}). \quad (2)$$

Here A denotes the area, N the number of repeating units, k is Boltzmann's constant, $\mathbf{r}_{i,j} = \mathbf{r}_i - \mathbf{r}_j$ is the difference between the projections onto the plane of the head groups of the position vectors $\mathbf{r}_i$ and $\mathbf{r}_j$ of two repeating units i and j , $\mathbf{a}_{i,j}$ their relative

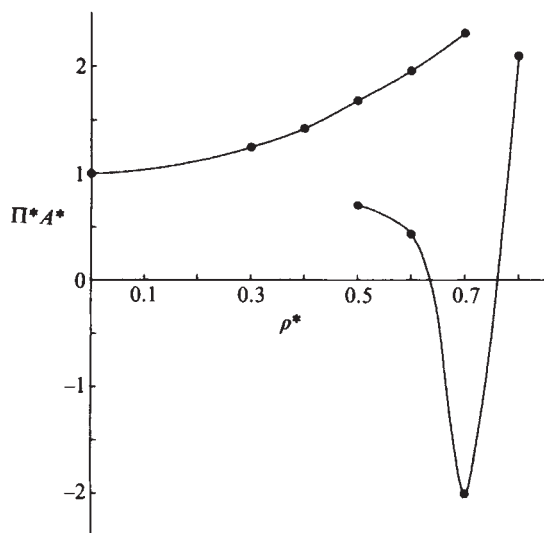


Fig. 2 Lipid monolayer isotherms at temperatures above and below the critical temperature. The upper curve corresponds to $T^* = 1$, the lower one to $T^* = 1/15$. The product $\Pi^* A^*$ equals the left hand side of equation (2).

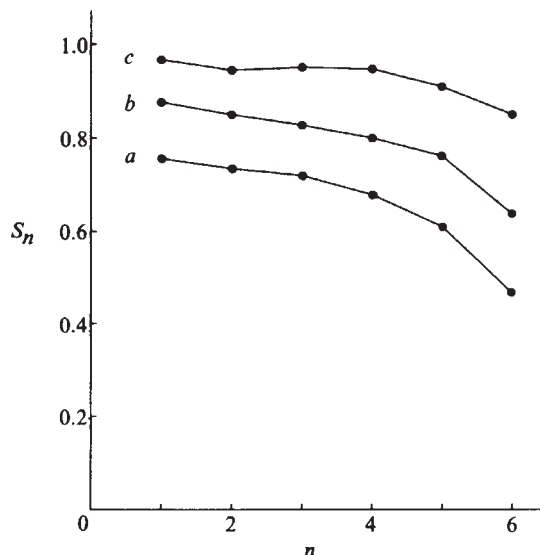


Fig. 3 Lipid monolayer order parameters at $T^* = 1$ and $\rho^* = 0.3$, 0.5 and 0.7 (curves a, b and c, respectively).

acceleration in the plane of the head groups and $\langle v^2 \rangle$ the average of the squares of the projected velocities. The time step used in the integration procedure was chosen as $0.005 \times \sigma(m/kT)^{1/2}$, with m the mass of a repeating unit. Averages were taken over 24,000 time steps. In this time a repeating unit covered a mean distance of approximately 200σ . Figure 2 shows a number of points belonging to two isotherms. The isotherm corresponding to the lower temperature shows the characteristic part of the Van der Waals loop which indicates the coexistence region of a system with a first order phase transition. The occurrence of this loop is interpreted to mean that the isotherms of the lipid monolayer are flat in the coexistence region. As this phase transition takes place at rather low densities, it corresponds to the transition between the liquid expanded and gaseous states of the monolayer.

Figure 3 shows the density dependence of a set of important parameters—the order parameters S_n , which measure the degree of order in the conformation of a chain. These quantities are defined by the relationship

$$S_n = \frac{3}{2} \langle \cos^2 \theta_n \rangle - \frac{1}{2} \quad (3)$$

in which the brackets denote an average over all chains and θ_n the azimuth angle of the n th bond, minus its value in the all-*trans* state, measured with respect to the chain axis in this particular state (see discussion in ref. 8). Both the way in which the order parameters depend on the density and their numerical values agree with the results of Scott², who used the Monte Carlo method. Knowledge of the order parameters is important in mean field theories for lipid monolayers of the type discussed by Marcelja⁵.

The system discussed here could quite easily be treated numerically. This justifies the expectation that the model can be extended in several ways. We suggest the following possibilities. (1) Extending the number of chains and increasing the number of repeating units per chain. (2) Incorporating the energy difference between a *trans* and a *gauche* configuration, so that it will be possible to study the main phase transition of the monolayer, which separates the liquid-condensed and liquid-expanded states. (3) Adding one or more proteins to the monolayer.

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The quasi 2-day wave in the Southern Hemisphere mesosphere

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We report here preliminary results of a coordinated series of observations made in January and February 1980 to observe the quasi 2-day wave in winds in the Southern Hemisphere mesosphere. The observations were made with the aim of measuring both the vertical and horizontal structure of the wave whose presence has previously been observed at several meteor wind stations in the Northern Hemisphere and at one station (Adelaide) in the Southern Hemisphere^{1–3}. Rocket observations also indicate the possible existence of a 2-day wave in the winds near the equatorial stratopause⁴. The observations suggest that the wave is a late summer phenomenon, peaking in late July/early August in the Northern Hemisphere and in late January/early February in the Southern Hemisphere.

Quasi 2-day waves with amplitudes of the order of 10–20 m s⁻¹ appear to be typical of both meridional (NS) and zonal (EW) winds in the mid to high latitudes in the Northern Hemisphere⁵, although amplitudes nearer 30 m s⁻¹ have been quoted for NS oscillations near the Equator⁶. However, an examination of a decade (1966–75) of Adelaide meteor wind data shows amplitudes as large as 50 m s⁻¹ in the NS wind component. Further investigations are therefore required to see if the differences in amplitude are due to hemispheric or latitudinal differences or both. Furthermore, more precise measurements of the wave period are required if the source of the wave is to be determined, because many of the previous investigations were for durations of the order of 5 days. Periods in the range 48 ± 6 h have been quoted from the Northern Hemisphere results but a period near 51 h seems favoured^{1,7}.

Our observations were made from three stations—Townsville (19°40'S, 146°50'E) and Adelaide (34°35'S, 138°30'E) situated in northern and southern Australia, respectively, and Christchurch, New Zealand (43°40'S, 172°40'E). The observations were made using the partial reflection drift technique which

enabled both the NS and EW winds to be measured over the 65–100 km height range at either 2 or 2.5 km height intervals (depending on the station) with a time resolution of less than 1 h (refs 8, 9). For the results reported here, hourly mean values were used and only the 80–100 km region is considered because over this height range data are available for both day and night, enabling the tidal wind components to be easily extracted. Observations were made at Christchurch for the whole of January and February, at Adelaide for the period 10 January–12 February and at Townsville for the period 15–31 January. All the data were continuous except for a few occasions when data were lost due to power failures.

Figure 1 shows the hourly mean meridional winds at an altitude of 88 km slightly smoothed by a 3-h running mean, as observed at Adelaide and Townsville. It is apparent that a strong oscillation with about a 2-day period had begun by 10 January at Adelaide, but it did not reach full amplitude at Christchurch (not shown) until 12 January. The oscillation was at its most stable between 10 January and 6 February, and seemed to die out completely by 12 February. At all three stations the NS component of the oscillation was 2–3 times larger in amplitude than the EW component, and maximum amplitudes of up to 50 m s⁻¹ occurred in late January. These observations agree well with the previous results from the Adelaide radio meteor winds³.

As may be seen from Fig. 1, strong 24- and 12-h tidal components were superimposed on the quasi 2-day wave, as expected in view of previous observations at Adelaide. Because of the presence of these strong tidal components the data for each height were analysed with a least squares fitting process to find the mean amplitude and phase of the quasi 2-day wave as well as the period which best fitted the observations. The zonal (*u*) and meridional (*v*) wind components were separately represented as a function of time (*t*) by

$$u(t), v(t) = a_0 + \sum_{i=1}^3 a_i \sin\left(\frac{2\pi}{T_i}t + \phi_i\right)$$

where *a*₀ is the prevailing wind and *a*_{*i*} and *φ*_{*i*} give the mean amplitude and phase for the 12 h (*i* = 1), 24 h (*i* = 2) and the wave of period *T*₃ which was varied systematically between 46 and 52 h. We found that the best fit occurred for a wave period of 49 ± 1 h.

The amplitude of the 2-day wave seemed to vary with latitude. At Townsville and Adelaide the amplitudes of the meridional component had mean values of 30–35 m s⁻¹ in the 85–90 km region and decayed above 90 km. At Christchurch the amplitude was about 18 m s⁻¹ at 88 km and increased slowly with height to reach a value of about 25 m s⁻¹ at 98 km. The zonal wind components decayed with height at all three stations. The 2-day wave amplitudes were significantly larger than the tidal amplitudes. For example, at Adelaide the mean amplitudes were about 15–20 m s⁻¹ in the EW and NS components for both the 24- and 12-h tides. The prevailing winds were typical of a late summer circulation, that is, eastward at heights above 80 km and with a weak equatorward flow.

The time of maximum eastward and northward winds occurred at earlier times at the higher altitudes, implying a downward

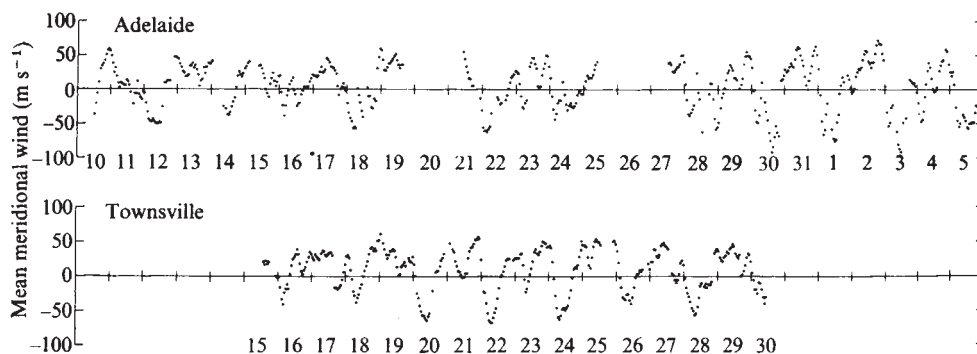


Fig. 1 Hourly mean meridional winds at 88 km observed at Adelaide (35°S) and Townsville (20°S) from 10 January to 5 February 1980, showing the 2-day wave. The values have been smoothed with a 3-h running mean.

phase and upward energy propagation. For the meridional component the implied vertical wavelengths were 50 ± 10 km at Townsville and 100 ± 10 km at both Adelaide and Christchurch. The phase of the EW oscillations seemed to lead the NS by 6–12 h. The NS oscillations at Adelaide and Christchurch showed a consistent 15 ± 1 h phase difference, with Christchurch leading. Assuming the phase difference to be due solely to the difference (34°) in longitude, these results are not inconsistent with a westward-propagating planetary wave of zonal wave number 3.

The 2-day wave observed here seemed to persist for ~13–15 cycles, longer than any previous 2-day wave observed at Adelaide. The period was closer to 48 h than the 51–52 h suggested by the Northern Hemisphere results. The amplitude variations with latitude suggest that the meridional velocity amplitude maximizes at low latitudes. It is still not clear whether the wave is larger in the Southern Hemisphere than in the Northern Hemisphere, although the amplitudes at Christchurch do seem to be slightly larger than the results reported from mid to high northern latitudes⁷. However, the inferred zonal wave number agrees with the values found in the Northern Hemisphere^{5,10}. Finally, we note that although the phase variations with height indicated that energy was propagating vertically, the little or no growth in amplitude with height indicates that this energy was being dissipated.

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Radiocarbon variations with the 11-year solar cycle during the last century

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As ^{14}C is produced in the atmosphere by interactions between neutrons and nitrogen atoms, changes in the cosmic ray flux associated with the 11-yr solar cycle will produce variations in ^{14}C production with the same period¹. These variations are difficult to observe because the atmosphere acts as a low-pass filter, greatly attenuating their amplitude². Previous investigations on short-term ^{14}C variations in tree rings either reported no correlation with the 11-yr solar cycle^{3–5} or found a correlation with an average amplitude of 1.5 to 3% (refs 6–10). However, Baxter *et al.* reported an average amplitude of about 10% in tree rings^{11,12} and in samples of whiskies, wines and plant seeds¹³, and Lavrukhina *et al.*¹⁴ found a 10% correlation amplitude in wood samples. However, very precise ^{14}C tree-ring measurements reported more recently^{15–17} show only minor short-term ^{14}C variations. A comparison of results obtained by Stuiver¹⁵ and Baxter *et al.*¹¹ suggests that the larger ^{14}C fluctuations found by the latter group may be due to a lower precision of their ^{14}C measurements. Here we have dated wines from the period 1909–52 and our results show ^{14}C variations with an average amplitude of $4.3 \pm 1.1\%$ during the 11-yr solar cycle.

Georgian wine samples of known origin (~38° N, ~45° E, altitude ~300 m above sea level), obtained from the Tbilisi Wine Museum, were used for ^{14}C measurements. The ^{14}C concentration of the alcohol fraction of wine should be closely related to the ^{14}C concentration of the grapes from which it was made, and thus to the atmospheric ^{14}C concentration. Our previous investigation of wines from the period 1950–75 showed a very good correlation between ^{14}C levels in the atmosphere and those in wine samples¹⁸. The use of wines allows an independent determination of the ^{14}C level. Because there is uncertainty regarding possible radial transport of ^{14}C between individual tree rings, the use of wines is preferable for investigating short-term ^{14}C variations during the last century. We also tried to use brandy samples, but found that these were unsuitable because of the special methods used in their preparation.

The methodology of alcohol extraction from wines as well as the method of acetylene and benzene preparation and counting have been described elsewhere¹⁸. The results are listed in Table 1 and plotted in Fig. 1 as $\Delta^{14}\text{C}$ values, defined by¹⁹

$$\Delta^{14}\text{C} = \delta^{14}\text{C} - (2\delta^{13}\text{C} + 50) \left(1 + \frac{\delta^{14}\text{C}}{1,000} \right) \%$$

$$\delta^{14}\text{C} = \frac{A - A_{\text{STD}}}{A_{\text{STD}}} \times 1,000\%$$

where A_{STD} is equal to the product of 0.95 and the activity of NBS oxalic acid, corrected for age to 1950, and A is the activity of the sample, also age corrected to the year of growth. A ^{14}C half life of 5,730 yr has been used in these corrections. Unfortunately, we were unable to carry out mass spectrometric measurements on these samples. However, our previous results²⁰, obtained with 15 samples of modern wines, showed that $\delta^{13}\text{C}$ values did not differ by more than $\pm 1\%$ from the mean value of -23.4% (1σ error of a single determination was 0.3%). The results given in Table 1 were corrected for isotopic fractionation using the assumed $\delta^{13}\text{C}$ value of -23.4% . The standard deviations quoted (1σ error) describe only uncertainties associated with the sample, standard and background determinations.

The cyclic trend in the ^{14}C data shown in Fig. 1 is very clear. The gradual decrease in ^{14}C concentration is due to the Suess effect. Our results, when compared with ^{14}C measurements in tree rings for the same period^{10,16}, are higher by about 5%. This is perhaps due to the remoteness of the wine growing area from industry and hence a less marked Suess effect.

For a better evaluation of short-term ^{14}C variations, we performed a spectral analysis of our ^{14}C values and compared our results with those of a spectral analysis of sunspot numbers

Table 1 Radiocarbon content of wines calculated with a half life of 5,730 yr

Year of growth	$\Delta^{14}\text{C}$ in wines (%)	Year of growth	$\Delta^{14}\text{C}$ in wines (%)
1909	$+0.4 \pm 2.4$	1931	-21.1 ± 1.6
1910	$+1.0 \pm 2.3$	1932	-17.3 ± 2.7
1911	-0.1 ± 2.5	1933	-20.5 ± 3.6
1912	-0.9 ± 2.6	1934	-18.0 ± 3.8
1913	-0.4 ± 2.6	1935	-17.3 ± 4.0
1914	$+1.8 \pm 2.3$	1936	-14.6 ± 1.9
1915	$+4.6 \pm 2.5$	1937	-16.9 ± 3.0
1916	$+4.3 \pm 2.1$	1938	-15.9 ± 2.6
1917	-0.3 ± 2.4	1939	-27.9 ± 3.2
1918	-2.5 ± 2.6	1940	-31.1 ± 2.4
1919	-7.6 ± 2.6	1941	-31.1 ± 3.1
1920	-8.7 ± 2.5	1942	-33.3 ± 3.8
1921	-8.8 ± 4.2	1943	-22.5 ± 4.1
1922	-8.5 ± 2.4	1943	-24.3 ± 2.3
1923	-10.1 ± 2.4	1944	-32.4 ± 2.2
1924	-7.3 ± 2.7	1945	-33.3 ± 2.0
1925	-6.8 ± 2.7	1946	-32.2 ± 2.9
1926	-5.2 ± 2.2	1947	-27.3 ± 3.7
1927	-5.5 ± 3.0	1948	-26.5 ± 2.9
1928	-6.5 ± 3.4	1949	-31.0 ± 2.6
1929	-9.7 ± 3.6	1950	-30.5 ± 2.3
1930	-3.6 ± 6.3	1951	-34.8 ± 2.4
1930	-11.1 ± 2.4	1952	-31.8 ± 2.0

Fig. 1 Radiocarbon data of Georgian wine samples ($\sim 38^\circ \text{N}$, $\sim 45^\circ \text{E}$) for the period 1909–52. The sunspot record has also been included for comparison.

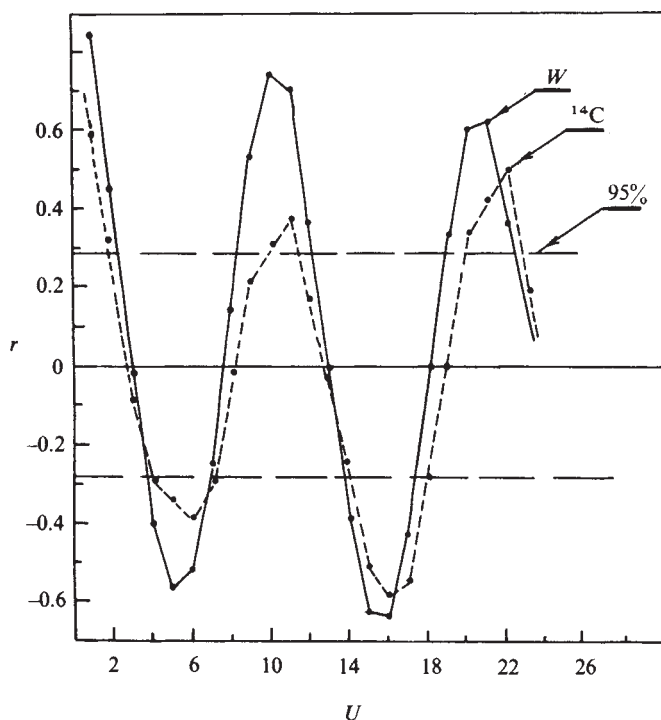
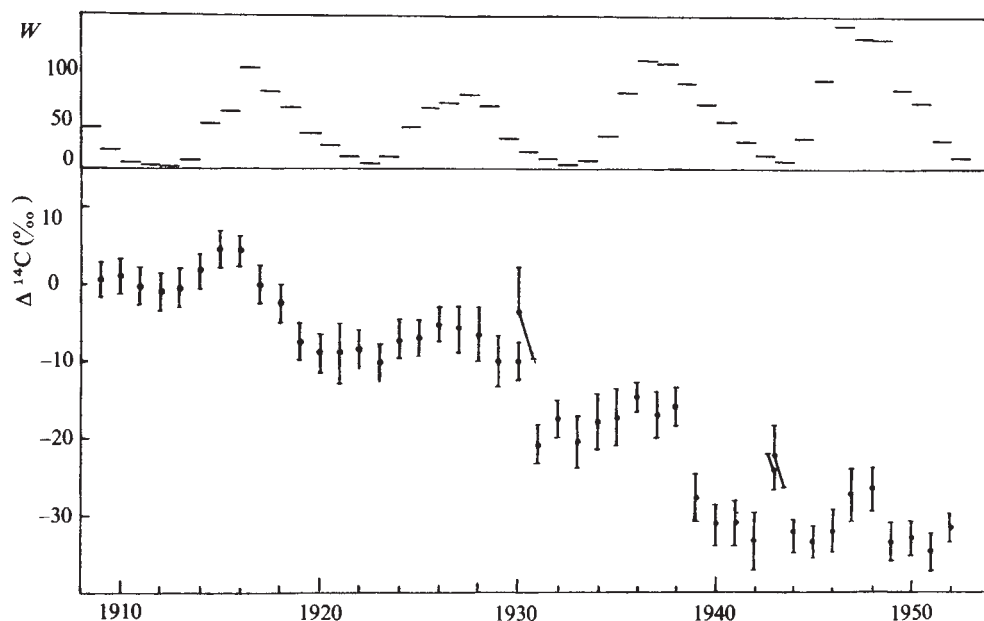


Fig. 2 Autocorrelation functions $r(U)$ of stationary time rows $\Delta^{14}\text{C}(t)$ and $W(t)$ for argument values $U_{^{14}\text{C}} = 11 \pm 1 \text{ yr}$ and $U_W = 10 \pm 1 \text{ yr}$.

W (Fig. 2). It can be seen that the autocorrelation functions $r(U)$ of stationary time rows $\Delta^{14}\text{C}(t)$ and $W(t)$, for argument values $U_{^{14}\text{C}} = 11 \pm 1 \text{ yr}$ and $U_W = 10 \pm 1 \text{ yr}$ have a quasiperiodical behaviour, with the period of 11-yr at the 95% confidence interval. The 11-yr solar cycle spectra are in both cases very sharp and have a similar shape. The basic power of the spectra is concentrated on the frequency $2.9 \times 10^{-9} \text{ Hz}$, corresponding to a period of 11 yr.

Further, we calculated correlation coefficients for various phase shifts for the different solar cycles. The required phase shifts varied from 3 to 7 yr, with a mean of 4 yr for four solar cycles. The correlation coefficients for optimal phase shifts were 0.67–0.86 for the various solar cycles, and the mean correlation coefficient for the phase shift of 4 yr was -0.58 .

As spectral analysis of the ^{14}C data has shown the periodicity of the $\Delta^{14}\text{C}(t)$ row, we also carried out harmonic analysis of

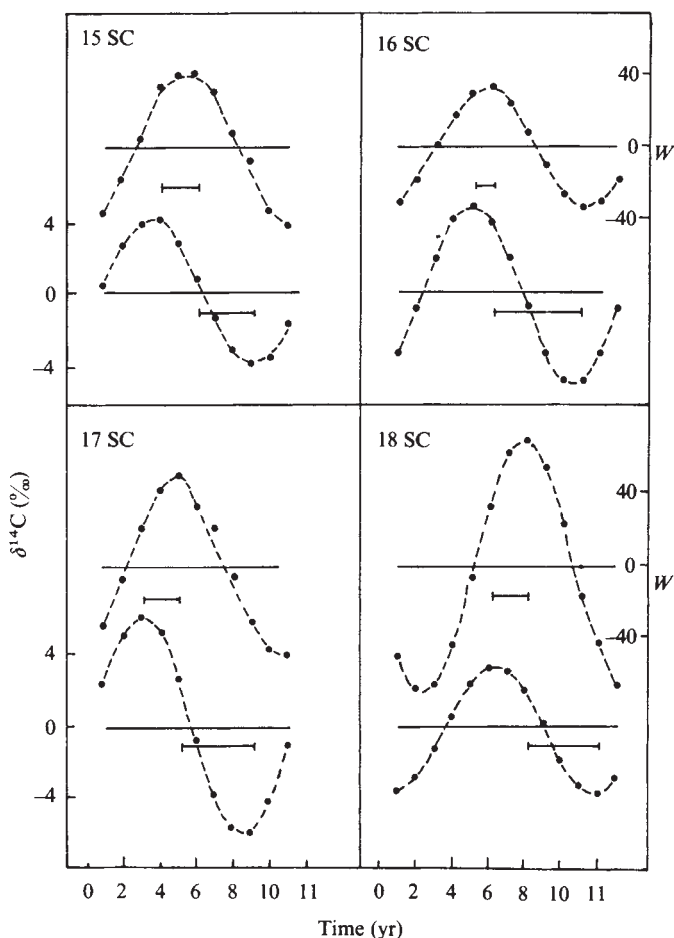


Fig. 3 The first harmonics of $\Delta^{14}\text{C}(t)$ and $W(t)$ for the 15th, 16th, 17th and 18th solar cycles (SC).

$\Delta^{14}\text{C}(t)$ and $W(t)$ rows. Figure 3 shows the first harmonics of $\Delta^{14}\text{C}(t)$ and $W(t)$ rows for different solar cycles. The amplitude of $\Delta^{14}\text{C}$ variations ranged from 3.3% to 5.6% for different solar cycles, and the average ^{14}C variation for the investigated time interval was $4.3 \pm 1.1\%$. The calculated time shift between W and $\Delta^{14}\text{C}$ maxima was 1–2 yr, and 3.5–5 yr between W maxima and $\Delta^{14}\text{C}$ minima for the different solar cycles. The harmonic analysis confirms the correlation analysis, especially that ^{14}C values lag behind Wolf sunspot numbers.

The amplitude of ^{14}C variations obtained in the present study is about a factor of 2 higher than the results of Damon *et al.*⁸ for the 18th solar cycle ($3.3 \pm 1.1\%$ compared with 1.5%), and 50% higher than our previous results for the 17th and 18th solar cycles¹⁰. Time shifts in the anticorrelation dependence of $\Delta^{14}\text{C}$ on W are comparable. The present results only partly cover the time interval investigated by Alekseev *et al.* (1890–1916)⁹, but except for the positive correlation, are similar for the years 1890–1900 (3% compared with our mean value of $4.3 \pm 1.1\%$). However, for the time interval 1900–16 the average amplitude obtained by these authors^{9,14} is about 10% compared with our value of $4.0 \pm 1.1\%$ for the 15th solar cycle. The reasons for the extreme cycles of 10% amplitude found in tree rings by Baxter *et al.*¹¹ and Lavrukhina *et al.*¹⁴ are poorly understood. Lavrukhina *et al.*¹⁴ found a sharp decrease of 3% per yr for the period 1909–16. This decrease may have been caused by an above average fossil fuel contribution.

More precise ^{14}C measurements in tree rings reported recently show only minor ^{14}C fluctuations during the 11-yr solar cycle^{15–17}. Furthermore, extensive ^{14}C series of single-year Douglas fir measurements¹⁵ have not shown statistically significant 11-yr periodicity between 1820 and 1950.

Thus, the present investigation has shown the existence of 11-yr ^{14}C variations in wine samples with a mean amplitude of $4.3 \pm 1.1\%$ over four solar cycles covering the period 1909–52. A comparison of our ^{14}C variations with those of previous investigations has revealed discrepancies which may be due to local variations of stratospheric inputs and the time lag between input and growing season. The observed ^{14}C variations may thus increase dating errors of short-lived samples used for archaeological research.

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Santorini tephra from Rhodes

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The large eruption of the Santorini (Thera) volcano, in ~1500 BC, inspired Marinatos to attribute the decline of the Minoan civilization to this eruption¹. It is still, however, being debated^{2,3} whether tsunamis were actually generated by the eruption of the volcano and whether the ash fall over eastern Crete was sufficient to cause its archaeologically attested desolation. Moreover, the problem of chronology remains unsolved. For, although there is no evidence that the eruption occurred after 1500 BC, the desolation of Crete is accepted as having taken place during the late Minoan IB period, at ~1450 BC (ref. 4).

We describe here recent excavations at Ialysos (Trianda) on Rhodes which have produced further evidence of the south-easterly distribution of the Thera tephra. Although a considerable amount of this tephra has been found there, this does not seem to have affected the continuity of life at the settlement, corroborating the view that the ash fall over Crete would have had little, if any, effect.

Confirming the wide distribution of tephra from the late Bronze Age eruption of the Santorini volcano in the south-east Aegean Keller has stated that: "still wanted for our problems is the distinct tephra layer in well defined late Minoan I stratified position"⁵. This tephra layer has now been located in Rhodes near the old Italian excavation at Trianda (Ialysos). We started an excavation on land in May 1978, to examine the possibility of building a new house there. The proximity of this land to the area which Monaco had excavated in 1935 and 1936^{6,7}, as well as some technical problems, meant that additional care had to be taken causing slow progress and delaying the start of the work. The pottery evidence (Fig. 2) made assigning the first architectural remains (Fig. 1) to the late Minoan settlement an easy task. These remains, at a depth of 1.5 m below the present surface, required careful excavation so that we could study the stratigraphy and thus help to solve the problems already posed by the excavation of the late Bronze Age city of Akrotiri on Thera⁸. As work progressed, to a depth of 1.80 m below the present soil surface, a layer of whitish powdery substance 10 cm thick was

Table 1 Mineralogical analyses* of samples from Rhodes

	Minoan pumice Thera (Ø4)	Minoan tephra Kos (Ø3)	Minoan tephra Rhodes (Ø8)
SiO ₂	70.76	71.25	71.26
TiO ₂	0.31	0.32	0.31
Al ₂ O ₃	2.06	2.02	1.97
MgO	0.33	0.31	0.31
CaO	1.43	1.37	1.30
Na ₂ O	4.64	4.42	4.51
K ₂ O	3.34	3.30	3.4
Cl	0.36	0.36	0.39
Total	96.93	97.13	97.06
H ₂ O (by difference)	3.0	2.9	3.4

* Analyses performed by J. Keller, Albert-Ludwig Universität, Freiburg.



Fig. 1 Theocharis' land, Trianda, Rhodes. A general view of the excavation.



Fig. 2 Pottery fragments from above the tephra layer.



Fig. 3 The tephra layer in square B3.

observed in squares B3–B4 (Fig. 3). The uncontaminated layer of this whitish substance covered $\sim 15 \text{ m}^2$, whereas almost the entire excavated area ($\sim 90 \text{ m}^2$) showed patches of this material mixed with earth. The first sample collected was sent to Indiana University, Bloomington, for determination of the refractive index. This material was identified as tephra from Thera on 20 October 1978. Eight more samples were later collected from almost all the excavated area (squares B3–B4: three samples; from just left of square A5 and from squares A6, B5, G6 and E3 one sample) and sent to Albert-Ludwig Universität, Freiburg, for mineralogical analysis. These results are shown in Table 1.

Having identified the tephra from Rhodes, it only remained to elucidate when this tephra came to the island. The only pottery evidence from beneath the clear tephra layer consists of sherds belonging to the same vase (Fig. 4). It has been difficult to look for more pottery underneath the pure tephra layer without destroying it. Thus this investigation has been postponed until all problems relating to the tephra layer have been solved. The style and decoration of the above vase place it in the late Minoan I A period^{9,10}. All the pottery fragments collected from above the clear tephra layer are mixed, dating from late Minoan I A to late Helladic II B (Fig. 2). Although there is little pottery evidence from under the tephra stratum, note that all the later pottery comes from above the tephra. This location of the later pottery mixed with late Minoan I A suggests that the tephra fall did not cause any significant break in the life of the settlement. This is of paramount importance as far as the eruption of the

Santorini volcano and its consequences are concerned. Because if the tephra at Trianda had been airborne, as is suggested by its fine quality and the purity of the layer, then:

(1) A considerable amount of tephra must have fallen over the south-east Aegean region and, anyway, more than that which fell on the eastern part of Crete, where it is estimated to have formed a layer $\sim 1\text{--}5 \text{ cm}$ thick¹¹.

(2) It is confirmed that northwesterly winds prevailing during the eruption sent the tephra south-east¹¹.

(3) It is also confirmed that Rhodes and the south coast of Turkey "have suffered severe tephra-fall"¹¹.

(4) Despite its thickness, the tephra which fell on Rhodes seems to have had very little if any, influence, on the settlement of Ialysos. This implies that the effect of the tephra fall on Crete was even less. Therefore, the problem of the cause of the desolation of eastern Crete^{12–14} remains unsolved, and the disruptive influence of the volcanic eruption of Thera on the development of Aegean civilization more doubtful^{15,16}.

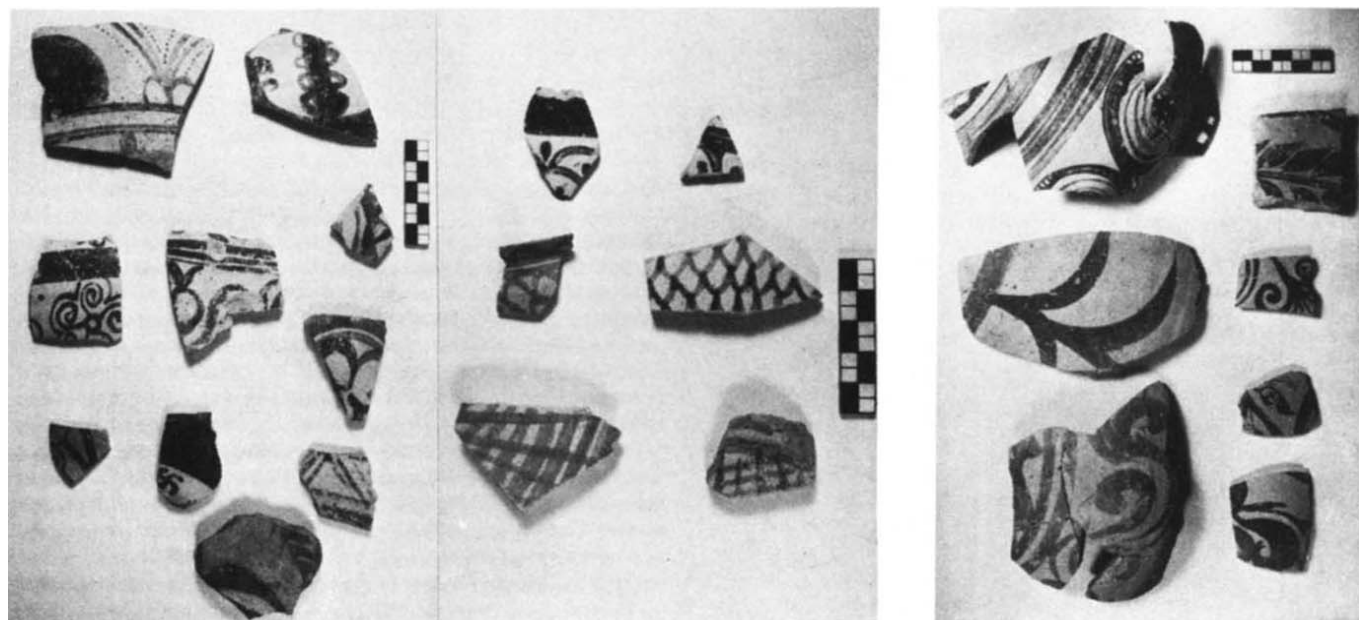


Fig. 4 Pottery fragments from underneath the tephra layer.

We dedicate this paper to the memory of the land owner Mr John Theocharis who died suddenly in the summer of 1979. We thank Professor C. Renfrew and Professor J. Luce for taking

care of the first sample when they visited the excavation. We also thank Professor Charles, Dr Dorothy Vitaliano and Professor J. Keller for the examination and analyses of the samples.

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Pollen analytical evidence for early forest clearance in North Sumatra

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Pollen analysis of sediments from the Toba Highlands of North Sumatra, Indonesia reported here suggests strongly that forest clearance by man began ~7,500 yr BP. Less convincing evidence suggests that man may have been disturbing the vegetation perhaps from ~17,800 yr BP, but climate has also changed and isolation of causal factors is, therefore, difficult. No Sumatran archaeological sites have yet been scientifically excavated, nor are ^{14}C dates available¹. However, megaliths possibly 2,000 yr old occur in the study area² and Hindu-Buddhist remains occur further south². Lowland shell-middens may be much older^{1,3}. Forest clearance may have begun by 11,000 yr BP on Taiwan⁴ and agriculture between 14,000 and 8,000 yr BP in Thailand^{5,6} but these conclusions have been disputed^{7,9}. Archaeological evidence for agriculture dating from ~9,000 yr

BP onwards has been found in Papua New Guinea^{10,11} while rice may have been cultivated in Sulawesi¹² by 6,000 yr BP.

Little palynology has been undertaken in Indonesia¹³⁻¹⁵. A 9.76-m long core from Pea Sim-sim swamp (Fig. 1) has yielded a basal ^{14}C date of $18,496 \pm 95$ (SSR 472). The site is at an altitude of 1,450 m on a plateau (mainly deforested) about 30 km west of an escarpment which falls sharply to Lake Toba at 900 m. Mountains rising above 2,000 m occur 40 km to the north-west, east and south-east. Swamp and limnic sediments have infilled a basin ~100 m in diameter, probably volcanic in origin. Volcanic sediment is absent from the site stratigraphy but the plateau itself is covered with rhyolitic ash at least 75,000 yr old¹⁶ from which the soils are derived.

Climatic data are not available from the immediate locality but readings from Siborongborong¹⁷ nearby indicate a mean annual rainfall of 2,000-2,100 mm for the region. The occurrence of lower montane forest in places demonstrates that the plateau is capable of supporting well developed vegetation in existing conditions. Much of the Toba Highlands, including the area studied, is under wet or dry rice cultivation but millets and, less frequently, taro and sweet potato are also grown.

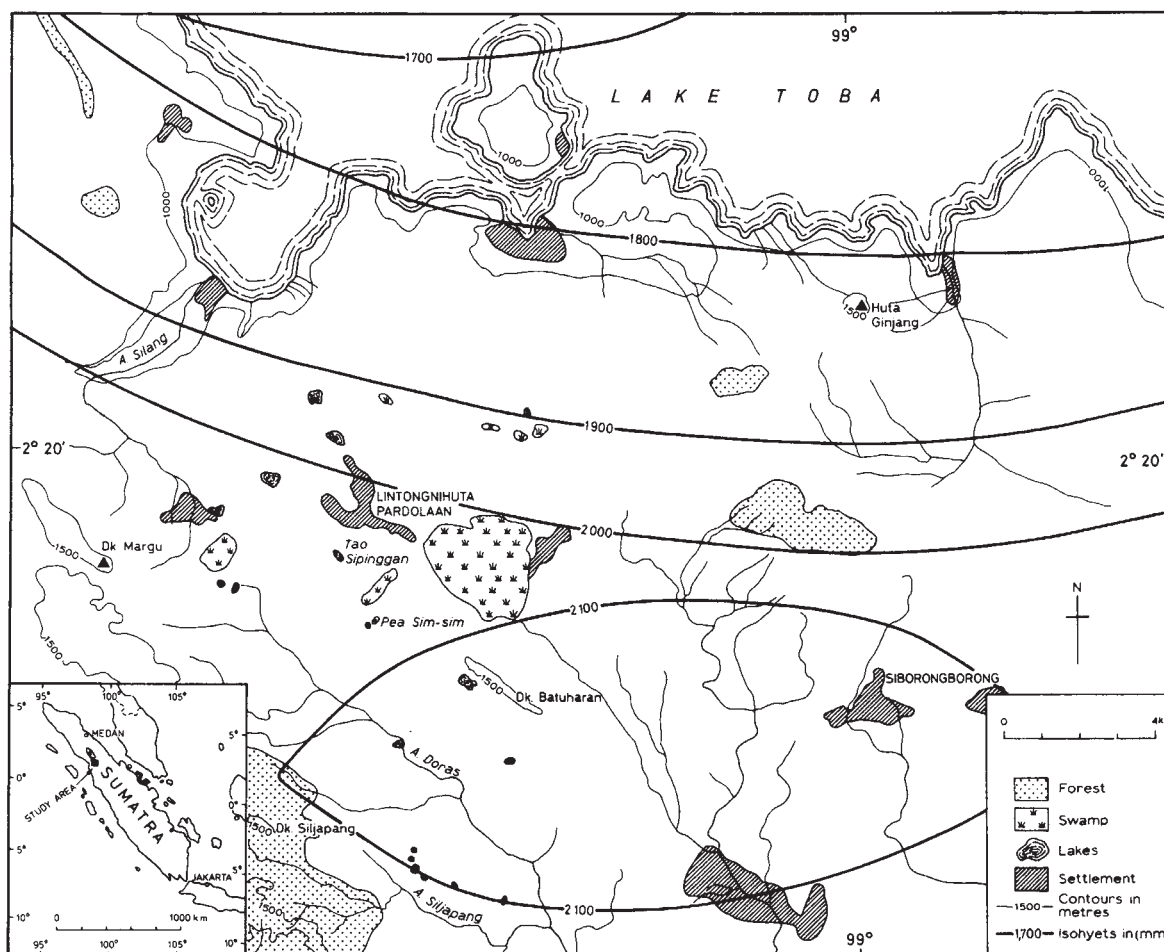


Fig. 1 Location map incorporating topographical data from Hind 1063, sheet 37, third edition and isohyets constructed using ref. 17.

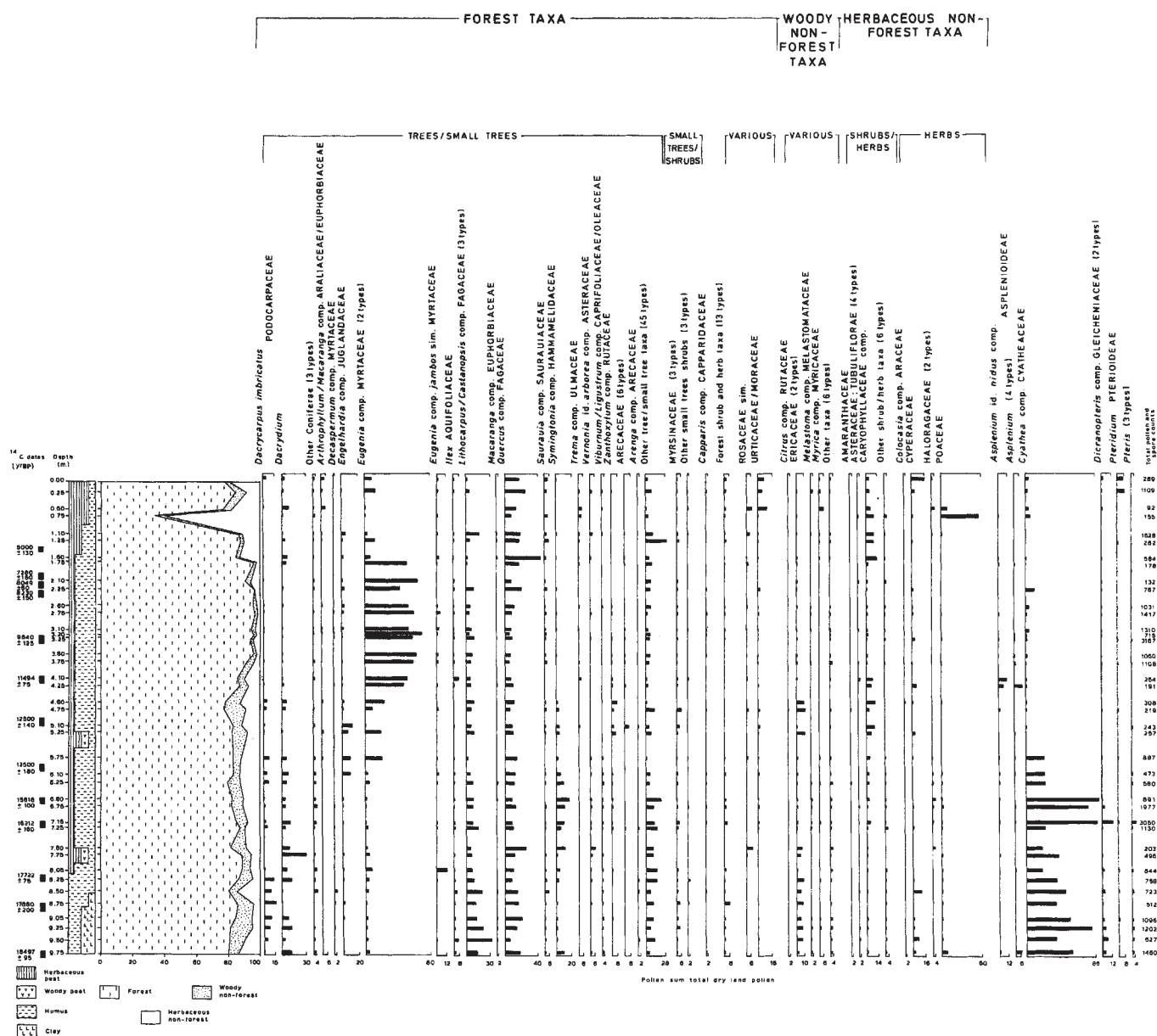


Fig. 2 Pollen diagram and site stratigraphy of the Pea Sim-sim site.

The core analysed was extracted from near the swamp centre. Unfortunately the bottom of the deposit could not be reached. Figure 2 shows a pollen diagram based on an ecological grouping of those pollen taxa which were determined and selected indicator pollens. Radiocarbon dates have been obtained for levels where the biostratigraphy shows important changes and the pollen diagram has been interpreted using spectra from surface sediments, an ecological survey and reference to literature on South-east Asian botany¹⁸⁻²⁵.

Figure 2 can be divided into four sections. The lowest part, dating between ~18,500 yr BP and an estimated 16,500 yr BP, is dominated by Coniferae and Fagaceae although *Symingtonia* is important in the basal sample. Comparison with the surface sample from the core suggests that forest pollen is over-represented. Indicators of open conditions occur but definite alpine taxa are absent. *Lithocarpus/Castanopsis* pollen is generally deposited within or near the forest but *Quercus* and, particularly, Coniferae pollen seems to be much more widely dispersed. Most of the forest pollen may have been transported in from lower altitudes, however, a mosaic of alpine and upper montane forest vegetation could have been present on the

plateau. A brief decline in forest pollen around 17,800 yr BP may relate to climatic deterioration, man's influence, or both: the occurrence of a high percentage of *Cyathea* spores in the lower part of Fig. 2 could be attributed to disturbance by fire while the rapidity of sedimentation supports the view that open conditions existed.

Lithocarpus/Castanopsis or Fagaceae (with *Quercus*)-*Symingtonia* forest became established at ~16,500 yr BP. This persisted until around 12,000 yr BP and may indicate climatic amelioration. *Symingtonia* can grow between 1,000 and 3,600 m above sea level. It is commoner above 1,700 m in Central Sumatra now but its natural lower limit in North Sumatra is not known. Fairly high percentages of taxa which can be significant in secondary forest and swamp forest in addition to primary forest are present and Caryophyllaceae have some of their highest percentages in the core. More ecological data are needed to clarify whether man may have been disrupting the vegetation.

The third part of Fig. 2 dates between ~12,000 and 7,500 yr BP (estimated). *Eugenia* attains large values in many levels. *Lithocarpus/Castanopsis* is quite important but Coniferae and

Symingtonia decline. The change could be attributed to altering climate, with upper montane forest giving way to lower montane forest, but *Eugenia* is often a component of swamp forest and the site stratigraphy indicates that swamp forest probably did exist in the past. As no swamp forest remains in the region it has not been possible to assess the efficiency of the formation in filtering the atmospheric pollen rain. The probability that *Eugenia* pollen especially is over-represented has to be borne in mind while the likelihood that the taxon was growing on dry land and the swamp complicates the interpretation. A *Lithocarpus/Castanopsis-Eugenia* forest may have been present on dry land.

Pollen of well dispersed taxa (*Dacrydium*, *Quercus* and *Urticaceae/Moraceae*) increases in the top section of the core while *Symingtonia* and *Lithocarpus/Castanopsis* pollen decreases. This suggests an opening out of the forest. *Eugenia* declines abruptly to fairly constant low levels after ~6,500 yr BP; possible disturbance indicators such as *Arthropodium/Macaranga* and *Trema* are commoner and the herbaceous non-forest component becomes especially significant. The suddenness of the change suggests that man was probably responsible. Perhaps the swamp forest was the major remaining source of wood? Had swamp forest not been so prominent, earlier dry land forest clearances might have been more strongly shown in the pollen stratigraphy.

Cereals may have been cultivated throughout much of this phase but work on the grass pollens proved to be inconclusive. However, the early literature²⁶⁻²⁸ describes a land largely deforested and planted with rice, much as it is now.

Pollen of other possible cultivars (particularly of fruit trees) occurs in numerous samples but the level of pollen identification and statistical considerations make any definite pronouncements unwise.

These findings suggest that agriculture might have been practised in Sumatra for much longer than was previously suspected, but further palaeoenvironmental research is desirable for verification, elaboration and assessment of possible regional interactions.

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Periodical cicada nymphs impose periodical oak tree wood accumulation

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All deciduous woody tree species of the eastern United States serve as hosts for large numbers of root parasitizing cicada nymphs (*Magicicada* spp.)¹⁻⁴. Periodical cicadas spend their larval lives 6-24 inches underground, feeding on xylem fluid from rootlets and roots⁵. They emerge every seventeenth year in the north or every thirteenth year in the south. Densities of cicadas underground are very great—Dybas and Davis report emergence densities of over 300 nymphs per square yard or about 1,500,000 per acre. This represents the highest reported biomass values of any naturally occurring terrestrial animal⁶. During the adult stage, which lasts 3-4 weeks, cicadas mate and females lay their eggs in twigs of deciduous trees. When the eggs hatch the first instar nymphs fall to the ground to begin their 17-yr development. The damaging effects to twigs by ovipositing adult cicadas is well established⁷⁻¹³ but the major effect of cicadas may be due to the feeding of nymphs: nymphs are implicated in a reduction in productivity of orchard trees¹⁴⁻¹⁶. However, no studies have compared growth of parasitized and unparasitized trees in the same environment. I report here that feeding cicada nymphs can reduce tree growth, as measured by growth rings, by as much as 30%.

Fifty scrub oak trees (*Quercus ilicifolia*) were selected randomly on a flat plain of sandy soil, 3 km east of Ridge, New York¹⁷. Mature periodical cicadas, *Magicicada septendecim* (Brood XIV) had emerged there in 1974 (refs 17-19). All the trees were about 1.5 m tall and grew in a nearly monospecific stand. To minimize environmental variability, trees were selected which did not border trails or roads and were not near runoff channels in the soil. The stems were cut one inch above the ground and annual wood accumulation was measured with a dissecting microscope and ocular micrometer for each year from 1978 to 1971. Although the trees are older than this, 1971 was as far back as it was possible to measure accurately radial wood increment. When cicada eggs hatch the first instars fall into the ground below the egg nest. As cicada nymphs do not travel far during their tenure underground, oviposition scars provide a reliable indicator of the number of nymphs in that immediate vicinity^{18,20}. Of the 50 trees selected, 20 had three or more egg nests (oviposition of one set of eggs by one female, mean = 7.20 ± 0.76 nests per plant), 15 had one or two egg nests and 15 had none. A mean of 19 eggs successfully hatch from each nest²¹. Infested and uninfested trees were in a random mixture on the site, precluding the possibility of an environmental cause for differential growth. Infested trees at this site support fewer cicadas than trees at other sites⁶. However, the dwarf oaks at this site are extremely small, are rooted in poor sandy soil and are very slow growing (Table 1).

For each of the three years before the emergence, no significant (0.05 level, Wilcoxon two-sample rank-sum test) difference was found between the trees with three or more egg nests and those with none (Table 1). During the emergence year and each of the four years following it, radial increment of the trees with no cicada eggs was significantly greater ($P < 0.05$) than trees with three or more egg nests. Trees with one or two egg

Table 1 Annual radial increment (mm) for scrub oak trees (*Quercus ilicifolia*) in Ridge, New York. Results of the Wilcoxon nonparametric two-sample test, the significance associated with each *U* value and the approximate *t_s* value are shown.

Year	3 or more egg nests		0 egg nests		<i>U</i> value from Wilcoxon two-sample test	Approximate <i>t_s</i> value	Significance
	Mean radial growth	Sample size	Mean radial growth	Sample size			
1978	0.23	20	0.35	15	206	1.87	0.05
1977	0.33	20	0.45	15	202.5	1.75	0.05
1976	0.31	20	0.41	15	212	2.07	0.05
1975	0.28	20	0.41	15	228.5	2.62	0.01
1974*	0.25	20	0.32	15	204	1.80	0.05
1973	0.26	20	0.28	15	165	0.50	none
1972	0.47	20	0.41	15	160	0.33	none
1971	0.78	20	0.58	15	190.5	1.35	none

*The emergence year.

nests were not significantly different than those without nests during any of the years and grew significantly more than trees with three or more nests during 1975 and 1977 only. In this analysis the three years preceding the emergence serve as a control. The fact that there was no difference in growth between the treatments for each of the three years preceding the emergence is evidence that microenvironmental factors, independent of cicadas, are not responsible for the observed growth effect during the years including and following the emergence.

It is difficult to interpret the reduction in growth of trees with egg nests during the emergence year. Cicadas emerge in May and June. The eggs hatch at least six to eight weeks after the emergence so that first instars do not enter the ground until July. It is expected that a large proportion of the annual wood increment would have already been added by this time. However, Phipps presents data showing that seasonal stem enlargement for *Quercus alba* varies considerably from year to year. A substantial fraction of growth may occasionally occur during July and August²². Current season wilting, death of branches, epinasty due to differential growth and short, weak shoots caused by oviposition wounds have been noted for *Quercus phellos* and *Quercus palustris*²³. Thus, oviposition damage or the large energetic demands made by last instar nymphs on their host plant before emergence, rather than feeding nymphs, could reduce radial growth during the emergence year. The data reported here do not allow testing of these hypotheses.

The previous analysis considered differences between trees of the three cicada treatments within each year. Next I examined differences within each treatment and preceding and following the emergence. Total radial increment was summed for the four years following the emergence (1975–78) and for the four years preceding and including the emergence (1971–74) for each tree. Those trees with three or more egg nests grew 43% more during the four years preceding the emergence than during the four years following it ($\chi^2 = 7.20$, $n = 20$, d.f. = 1, $P < 0.01$ using Friedman's method for randomized blocks)²⁴. No difference was found for the trees without egg nests ($\chi^2 = 0.70$, $n = 15$, d.f. = 1) nor for trees with one or two egg nests ($\chi^2 = 0.0$, $n = 14$, d.f. = 1). This result is unchanged if the data are analysed without the emergence year, 1974. This analysis shows an inverse relationship between a large number of cicada egg nests and radial incremental growth of *Q. ilicifolia* individuals following the emergence. In this analysis the trees without egg nests serve as controls. The fact that only those trees with three or more cicada egg nests in their canopies show a reduction in wood accumulation and those without cicadas show no effect is further evidence that climatic or other environmental factors are not responsible for the observed reduction in growth of trees with cicadas during the years following the emergence.

The result that cicada nymphs affect tree growth more in years including and following the emergence is in keeping with recent field observations. Periodical cicadas undergo five instars while

growing underground. Earlier instars feed on smaller rootlets²⁵. Younger nymphs live in short tunnels rather than the more permanent ellipsoidal cells inhabited by older nymphs^{25,26}. Young nymphs presumably change feeding sites each time the rootlet they are currently using dies²⁵. Older nymphs feeding on larger roots apparently do not kill their host root, resulting in relatively less damage to the tree by older nymphs^{5,25}.

The greater impact of feeding cicada nymphs in the years immediately following the emergence compared to the years immediately preceding the emergence may also be, in part, the result of a demographic reduction in cicada nymph density. Mortality factors such as predation by moles²⁷, direct competition^{27,28} and starvation²⁵ reduce the population of cicada nymphs. A schedule of survivorship for developing nymphs is needed.

Periodical cicadas occur in great numbers feeding on all species of deciduous trees in eastern North America. If the growth effect documented here is a general one, cicada nymphs are a serious pest. Feeding cicada nymphs are capable of reducing tree growth by as much as 30%. Because feeding nymphs decrease tree growth more during the years following an emergence than during the years preceding an emergence, they may be inducing a periodical pattern of tree growth.

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Sexual dimorphism in early anthropoids

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Sexual dimorphism in canine/premolar tooth size and in body size is found among many species of living primates and has been shown to be correlated with social organization. Among extant higher primate species that normally live in nuclear families consisting of a mated pair with their offspring, adult males and females are similar in body size and in the size of canine and anterior premolar teeth. In contrast, higher primate species living in more 'complex' polygynous groups (either single-male harems or multi-male groups) are characterized by sexual dimorphism in the size of canine/premolar teeth and frequently by body size dimorphism as well¹⁻⁴. We provide here the first evidence for sexual dimorphism in three species of primates from the Oligocene of Egypt—*Aegyptopithecus zeuxis*, *Propliopithecus chirobates*, and *Apidium phiomense*. This is the earliest record of sexual dimorphism among higher primates and suggests, by analogy with living species, that the earliest known fossil Old World anthropoids lived in polygynous (either single-male harems or multi-male groups) rather than monogamous social groups.

Four higher primate species have been recovered from the Upper Fossil Wood zone of the Jebel el Qatrani Formation in the Fayum Province of Egypt: two hominoids—*A. zeuxis*^{5,6} and *Pr. chirobates*⁵⁻⁸ and two parapiithecids—*Apidium phiomense*⁸ and *Parapiithecus grangeri*⁸. Until recently, most of these taxa were known from relatively few specimens preserving mainly molar teeth. However, recent cooperative expeditions by the Geological Survey of Egypt and Duke University have recovered more complete dental and cranial remains, as well as additional skeletal material of these early anthropoids^{9,10}. Thus, for the first time, there are numerous jaws preserving the lower canines and premolars (Fig. 1)—the teeth that are critical for identifying sexual dimorphism in higher primates^{11,12}.

The largest species from the Egyptian Oligocene is the early ape, *A. zeuxis*. The molar teeth of specimens assigned to this species are uniform in size; however there is a distinct bimodal distribution in the size of lower canines and premolars similar to that found in sexually dimorphic living species, with the larger canines and premolars presumably belonging to males and the smaller ones to females (Fig. 2a,b). In both sexes of *A. zeuxis*, the lower canine is a projecting subconical tooth and the anterior lower premolar is sectorial, with a large wear facet on the anterolateral surface where it occludes with the upper canine (Fig. 1a,b). The lower canines (Fig. 2a) and anterior lower premolars (Fig. 2b) of presumed females, however, are about 25% smaller than those of presumed males (Fig. 2a,b).

Propliopithecus chirobates is a smaller ape closely related to *A. zeuxis*. Although this species is known from fewer specimens than *A. zeuxis*, it shows a similar bimodal distribution of canine/premolar dimensions in conjunction with low metric variability in molar dimensions (Fig. 2a,b). As in *A. zeuxis*, these teeth are very similar in morphology in the two sexes (Fig. 1c,d), but differ in size.

In *A. phiomense*, a small parapiithecoid, the canine is not so

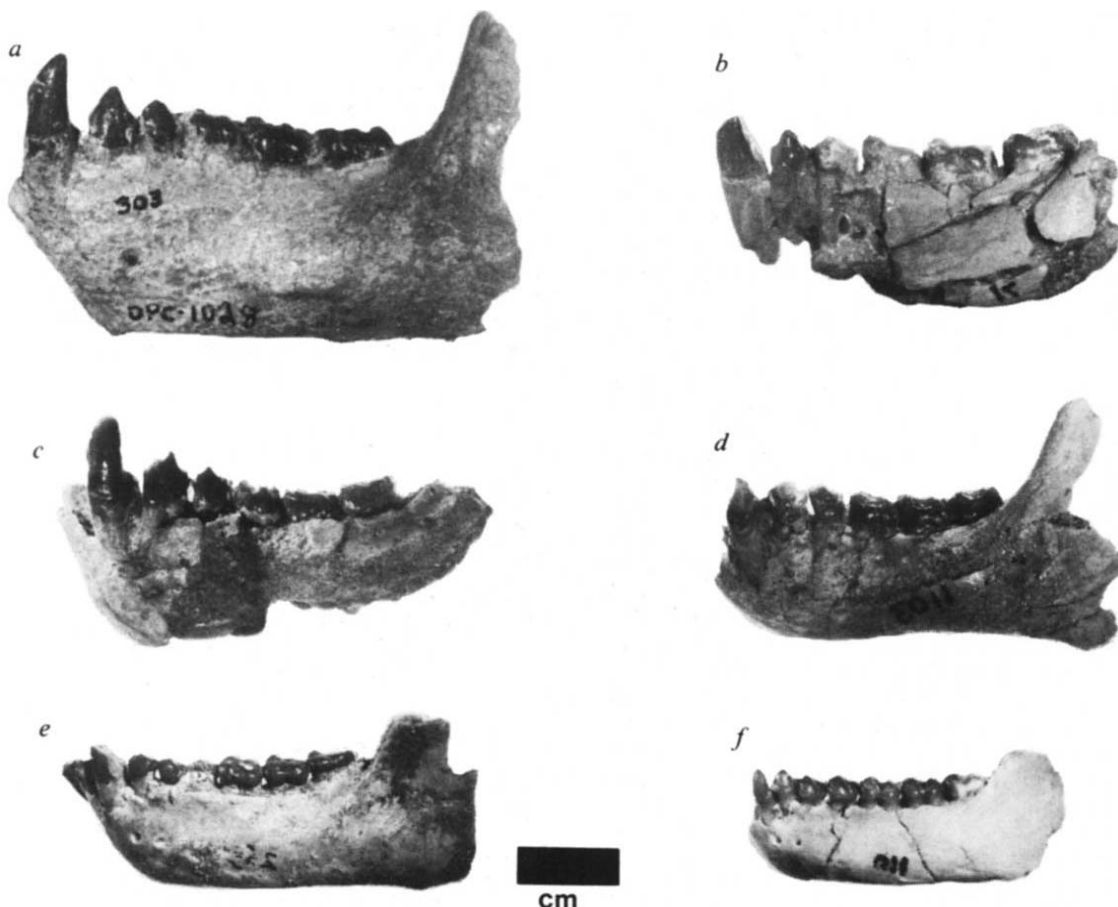


Fig. 1 Lower jaws of early Oligocene Anthropoidea from Egypt. a, *A. zeuxis*, male (DPC 1028); b, *A. zeuxis*, female (DPC 1042); c, *P. chirobates*, male (DPC 1029); d, *P. chirobates*, female (DPC 1103); e, *A. phiomense*, male (DPC 1089); f, *A. phiomense*, female (DPC 1104). All fossils are to the same scale.

Table 1 Mandibular depth, body mass ratio of adult males to adult females and social structure of anthropoidea

Species (N)		c.v. of mandible depth	Male mass		Social structure
			Female mass		
<i>Cebus apella</i>	(20)	19.5	1.30 ¹³		MM†
<i>Alouatta seniculus</i>	(20)	19.4	1.41 ¹³		S or MM†
<i>Nasalis larvatus</i>	(18)	18.3	1.37 ²		MM ¹⁵
* <i>Aegyptopithecus zeuxis</i>	(10)	16.3	*		*
* <i>Apidium phiomense</i>	(22)	13.6	*		*
<i>Macaca fascicularis</i>	(12)	12.6	1.16 ²		MM†
<i>Gorilla gorilla</i>	(40)	12.2	1.28 ²		S or MM ¹⁵
<i>Pan troglodytes</i>	(26)	9.2	1.04 ²		MM ¹⁵
<i>Presbytis rubicunda</i>	(12)	8.3	1.03 ²		S ¹⁷
<i>Hylobates lar</i>	(12)	8.2	1.03 ²		Mo†
<i>Saimiri sciureus</i>	(18)	7.5	1.10 ¹⁴		MM†
<i>Pithecia pithecia</i>	(17)	7.4	1.0 ⁹		Mo†
<i>Aotus trivirgatus</i>	(17)	7.0	1.04 ²		Mo ¹⁶
<i>Hylobates hoolock</i>	(16)	6.9	1.02 ²		Mo ¹⁸
<i>Symphalangus syndactylus</i>	(10)	5.9	1.03 ²		Mo ¹⁶

Mandibular depth was measured under M₁, sample sizes are to the right of the species. Social structures are categorized as follows: MM, groups with several adult females and several adult males; S, groups usually with one adult male and several adult females; MO, monogamous groups with one adult male and one adult female. All groups with offspring.

† Personal observation.

projecting and the shearing between the upper canine and anterior lower premolar (P₂) is not as well developed as in the hominoids (Fig. 1e, f). There is, nevertheless, dimorphism in the size of the lower canine relative to molar dimensions (Fig. 2a). At present, too little is known of the dental morphology of *Parapithecus grangeri* to determine the extent of sexual dimorphism in this parapithecoid.

In addition to dental dimorphism, *A. phiomense* and *A. zeuxis* also show evidence of dimorphism in the depth of the mandibular corpus. (There are not enough jaws of *Propliopithecus chirobates* or *Parapithecus grangeri* to allow statistical comparison of mandibular dimensions.) Among extant higher primates showing sexual dimorphism in body size, the smaller females have relatively shallow mandibles and the larger males have relatively deep mandibles. In species with little sexual dimorphism in body weight, there is no dimorphism in mandible depth. The coefficient of variation (100 s.d./ $\bar{x}$) provides an estimate of variability within a species that can be related to dimorphism, but does not require prior identification of the sex of a specimen¹¹. There is a very high positive correlation ($r = 0.940$) between the degree of sexual dimorphism in body weight ($\bar{x}$ female mass/ $\bar{x}$ male mass) and the coefficient of variation of mandible depth below the first molar (Table 1). *Aegyptopithecus zeuxis* and *Apidium phiomense* show high coefficients of variation for mandible depth suggesting considerable dimorphism in body size.

The correlation of social organization with canine/premolar dimorphism and body size dimorphism among living primates appears to be mainly the result of differences in intra-sexual competition and possibly also predator defense¹⁻⁴. In monogamous species, adults of both sexes appear to be equally active in repulsing same-sex intruders into the group and are often equally active in defense; thus there is little sexual dimorphism. In polygynous species, there is usually much more overt male-male competition for access to females and males often appear to play a greater role in troop defense; therefore males have large canines and anterior premolars and often greater size than females. The canine/premolar dimorphism and mandibular variation exhibited by *A. zeuxis*, *Propliopithecus chirobates* and *Apidium phiomense* are considerably greater than that found among any living monogamous higher primates and suggest that

all three extinct species were probably living in polygynous groups characterized by male-male competition.

Among living higher primates, nuclear families and associated sexual monomorphism are found among many of the more primitive New World monkeys and among the lesser apes, suggesting that a monogamous social structure is phylogenetically very old and is possibly the primitive condition for all higher primates¹⁹⁻²¹. The evidence of considerable sexual dimorphism in Oligocene hominoids and parapithecids presented here, however, indicates that more complex, presumably polygynous social structures are definitely very old, probably represent the primitive condition for Old World higher primates, and may well be primitive for all anthropoids.

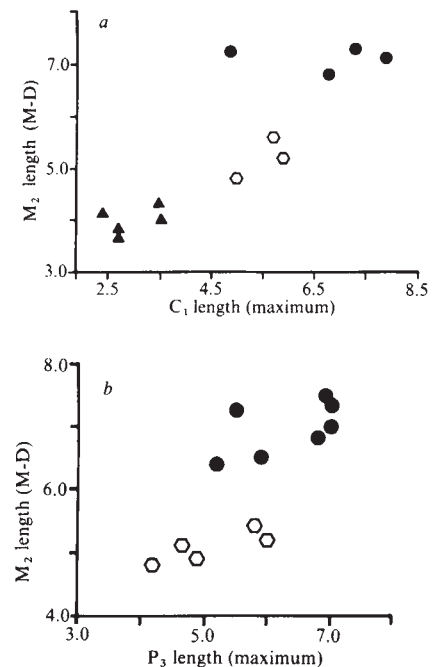


Fig. 2 a, Bivariate plot of length of C₁ versus M₂ for *Aegyptopithecus zeuxis* (●), *Propliopithecus chirobates* (○), and *Apidium phiomense* (▲). b, Bivariate plot of length of P₃ versus M₂ for *A. zeuxis* (●) and *Propliopithecus chirobates* (○).

Finally, the finding that sexual dimorphism is typical among Fayum apes has important implications for interpreting the phylogenetic status of *Propliopithecus haekeli*. Since its initial description in the early years of this century²², the type and sole specimen of this species has been put forth by many scientists as an early human ancestor because of its small canines²³⁻²⁶. To the contrary, the similarities between *P. haekeli* and females of *P. chirobates* indicate that *P. haekeli* is not hominid-like but rather is the female of a sexually dimorphic species^{5,27}. This invalidates the available evidence for an Oligocene divergence of humans and apes.

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Oxygen-linked CO₂ binding independent of pH in cephalopod blood

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The oxygen affinity of the haemocyanin-containing blood of some cephalopods is very dependent on the pH of the blood^{1,2}. The effect of pH on the oxygen affinity is expressed quantitatively as the slope of the function between $\log P_{50}$ and pH, where P_{50} denotes the oxygen tension at an oxygen saturation fraction of 0.5. This Bohr coefficient, $\Delta \log P_{50}/\Delta \text{pH}$, is about -1.8 in the case of *Loligo pealei*¹ and about -1.5 for *Sepia officinalis*². These pronounced Bohr effects are physiologically significant as they should allow the metabolically produced CO₂ in the tissues to promote the dissociation of oxyhaemocyanin and thus facilitate the unloading of oxygen to the tissues. However, the Bohr effect is tantamount to an oxygen-linked hydrogen ion binding to the respiratory pigment and the quantitative parameter of this Haldane effect is the change in the concentration of haemocyanin-bound hydrogen ion (ΔHcH) accompanying a change in the concentration of haemocyanin-bound oxygen (ΔHcO_2) at a constant pH. According to the linkage equation³, the Bohr coefficient and the Haldane coefficient are identical: $\Delta \log P_{50}/\Delta \text{pH} = (\Delta \text{HcH}/\Delta \text{HcO}_2)_{\text{pH}}$. This implies that in a homogeneous system, such as in cephalopod blood, the amount of protons bound to the haemocyanin upon deoxygenation from the fully oxygenated state, at constant pH, equals the negative value of the Bohr coefficient times the oxygen capacity. Thus, a Bohr coefficient of <-1 implies that more than 1 mmol of protons will be bound to the pigment per mmol of O₂ unloaded. Since the CO₂ produced in the tissues can maximally yield 1 mmol of protons per mmol of oxygen consumed, at a respiratory exchange ratio of unity, we reinvestigated the reciprocal effects of O₂ and CO₂ on the respiratory properties of cephalopod blood to see whether blood pH can decrease during O₂ unloading when CO₂ is the only 'proton source' and the Bohr coefficient is <-1.

A spectrophotometric determination⁴ of the oxygen affinity of the blood from *Sepia latimanus* at 25 °C gives the expression $\log P_{50} = -1.37 \times \text{pH} + 11.83$ in the pH range 7.3-7.7 and the Bohr coefficient of -1.37 means that this blood is suitable for our investigation.

The relationship between the CO₂ tension, pH and the total content of CO₂ in oxygenated and deoxygenated blood was determined by measuring both pH and the CO₂ content^{5,6} of blood brought into equilibrium with the appropriate gas mixtures in a tonometer kept at a constant temperature of 25 °C.

Figure 1a shows that the deoxygenated blood contains more CO₂ at a given CO₂ tension than the oxygenated blood, except at

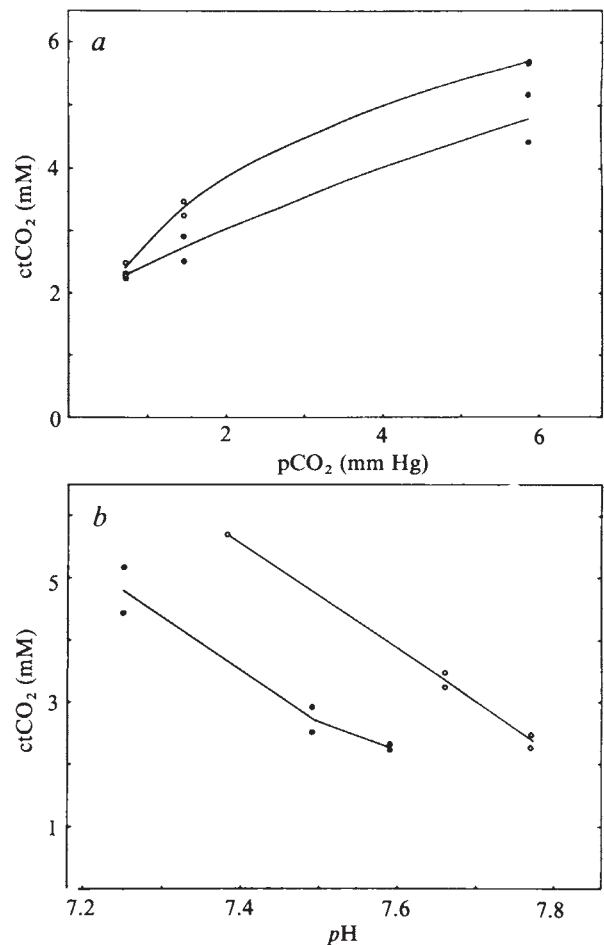


Fig. 1 a, CO₂ equilibrium curves of oxygenated and deoxygenated blood. ctCO₂ is the total CO₂ content. b, The data for total CO₂ content from a plotted against the corresponding measured pH values. Oxygen capacity = 1.86 mM. ●, Oxygenated; ○, deoxygenated.

the lowest CO₂ tension applied where the two curves coincide. As the blood has the same total CO₂ content (ctCO₂) at a CO₂ tension of about 0.7 mm Hg, irrespective of the state of oxygenation, one should expect the pH to be the same for oxygenated and deoxygenated blood. Figure 1b shows that this is not the case, as replottting the values for ctCO₂ from Fig. 1a against the corresponding pH of the blood reveals that a great pH difference exists between oxygenated (pH = 7.59) and deoxygenated (pH = 7.77) blood.

This pH difference at a constant CO₂ tension tells us that, as the total CO₂ is nearly unchanged, some of the CO₂ present in the oxygenated blood must have disappeared upon deoxygenation and that it has been replaced by an almost equal amount of bicarbonate.

To investigate this CO₂ component in oxygenated blood, CO₂ washout experiments were performed on oxygenated and deoxygenated blood. Blood tonometered to equilibrium at the lowest CO₂ tension was subjected to a CO₂ pressure of zero. Blood pH and ctCO₂ were measured repeatedly for about an hour and the results presented in Fig. 2.

The total content of about 2.3 mmol of CO₂ per l is in both cases washed out after 1 h, but both the time course and the total change in pH are different. The oxygenated blood shows a change in pH from 7.59 to nearly 7.74 and the change is almost fully established after 30 min when the system still contains about 1 mmol of CO₂ per l. By contrast, the deoxygenated blood exhibits a total change in pH from 7.77 to 7.99 and this change is complete after 70 min, when the CO₂ content of the blood is

reduced to nearly zero. The much smaller pH increase in the oxygenated sample compared with the deoxygenated sample and the time course of this change indicate that the CO₂ pool in the oxygenated blood contains a component of CO₂ that is liberated without a simultaneous call for protons, and that the size of this part of the total CO₂ is close to 1 mmol per l of blood.

We suggest that this CO₂ component is present in the form of molecular CO₂ bound to the oxygenated haemocyanin and that this CO₂ fraction is oxygen linked such that it is liberated when the oxygen is given off from the haemocyanin.

The size of the oxygen-linked CO₂ component can also be estimated if one assumes that the ctCO₂ of the deoxygenated blood, at the two highest CO₂ tensions applied, is composed solely of physically dissolved CO₂ (cdCO₂) and bicarbonate. The pK' values of the carbonic acid-bicarbonate system were calculated according to the equation:

$$pK' = pH - \log \frac{ctCO_2 - cdCO_2}{cdCO_2}$$

With the relationship between pK' and pH established, pK' values corresponding to the other measured values of pH for oxygenated and deoxygenated blood were determined and thereafter the apparent bicarbonate concentrations were calculated. The results of this procedure are represented in Fig. 3.

This plot shows that the calculated amount of apparent bicarbonate plus the physically dissolved CO₂ for oxygenated blood is between 0.7 and 1 mmol less than the measured values for total CO₂. Furthermore, it is now possible to test the identity between the values for the Bohr and Haldane effects. With an oxygen capacity of 1.86 mM (determined as half of the copper content of the blood) and a Bohr coefficient of -1.37, the total ΔcHcH at constant blood pH should be about 2.5 mmol per l of blood. When this value is compared with the increase in apparent bicarbonate at constant pH—2.7 mM at pH 7.38 decreasing to 2.5 mM at pH 7.59—a striking resemblance with

the predicted value above is apparent. The correspondence of these numbers adds validity to the procedure used concerning the calculation of the apparent bicarbonate concentration and to the estimate concerning the size of the oxygen-linked CO₂ component.

The effect of the oxygen-linked CO₂ component is a transformation of the Haldane coefficient to what could be called the functional Haldane coefficient, the latter expressed by the CO₂ binding potential of the system at constant pH. The size of this functional Haldane coefficient, in mM CO₂/mM O₂, ranges in *S. latimanus* from about 0.9 at pH 7.59 to about 1.1 at pH 7.38. This implies that no substantial decrease of the *in vivo* blood pH should occur when blood passes the tissue capillaries; on the contrary, small increases in blood pH should be more frequent, even at a respiratory exchange ratio of unity.

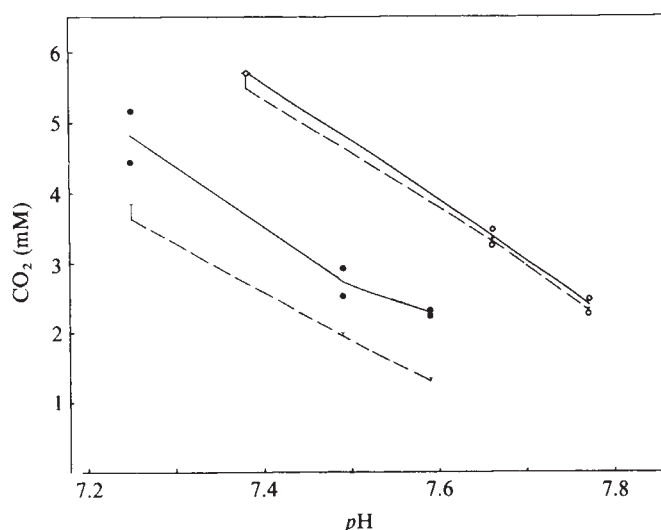


Fig. 3 This shows the same data as Fig. 1b, together with the corresponding calculated values for apparent bicarbonate concentration (broken line). Vertical bars (T) indicate the amount of physically dissolved CO₂ (solubility coefficient 0.039 mM l⁻¹ mmHg⁻¹). Oxygen capacity = 1.86 mM. ●, Oxygenated; ○, deoxygenated; —, ctCO₂.

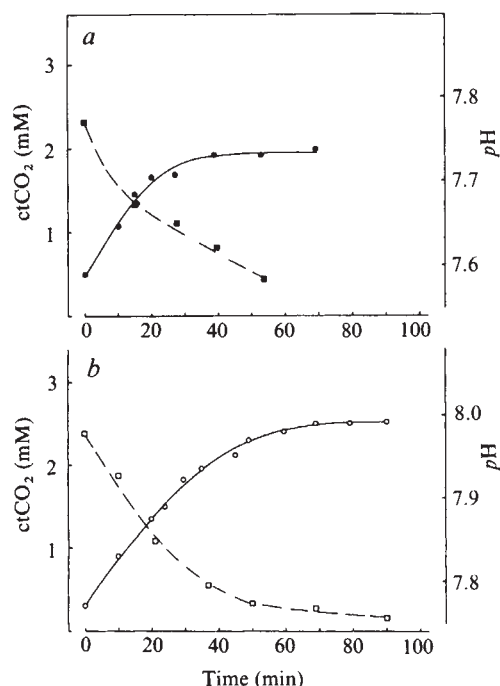


Fig. 2 CO₂ washout curves for a, oxygenated and b, deoxygenated blood. Squares, ctCO₂; circles, pH.

The question of whether pH can actually decrease during O₂ unloading may be answered affirmatively. A requisite for this answer must, however, be the presence of an oxygen-linked CO₂ component large enough to generate a functional Haldane coefficient: mM CO₂/mM O₂ at constant pH, well below unity. This condition is likely to be species dependent and related to metabolic rate and metabolic scope. In *S. latimanus*, a moderately active cephalopod, the condition is marginally present, but in the case of *L. pealei*, a far more active species, it is likely to be more developed.

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Isolation of human haematopoietic progenitor cells using monoclonal antibodies

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Studies of the differentiation of human haematopoietic cells have been greatly advanced by the use of *in vitro* cloning techniques to enumerate the myeloid and erythroid progenitor cells which form colonies in semi-solid medium¹⁻³. The granulocytic/monocytic colony-forming cells (CFU-GM) and erythroid burst-forming cells (BFU-E) are thought to be mononuclear cells, similar in size to medium size lymphocytes, but their ontogenetic relationship to other progenitor cells and the putative pluripotential stem cell remains obscure^{4,5}. This is partly because elucidation of parent-progeny relationships requires the isolation of precursor cells before they acquire mature phenotypic characters. Physical methods have failed to separate CFU-GM cleanly from other cells because of their heterogeneity, and immunologically specific markers for human progenitor cells have not been reported^{6,7}. We report here that a fraction containing all the granulocytic/monocytic and erythroid progenitor cells, as well as cells which may be lymphoid progenitors, can be isolated from bone marrow using a combination of monoclonal antibodies.

The anti-leukocyte antibody (anti-HLe-1) was prepared by hyperimmunization of BALB/c mice with human peripheral blood mononuclear cells. The immune spleen cells were fused using polyethylene glycol to NS-1 myeloma⁸ and the anti-HLe-1 clone was isolated by limiting dilution. By indirect immunofluorescence the antibody stains brightly all peripheral thymus-derived (T) and bone marrow-derived (B) lymphocytes and the majority of thymocytes. Peripheral blood monocytes and granulocytes and some bone marrow cells are weakly positive, whereas all other cell types tested, including mature erythrocytes and platelets, are negative^{9,10}.

The anti-myeloid antibody (TG-1) was derived, by limiting dilution cloning, from a fusion for which BALB/c mice were immunized with a glycoprotein fraction of thymocyte membranes (P.C.L.B. and A. Sullivan, unpublished observations). This antibody recognizes an antigen present on peripheral blood polymorphs and eosinophils but not on T and B lymphocytes and erythrocytes or the surface membrane of thymocytes. In bone marrow it labels predominantly cells of the granulocytic lineage. We do not know why we were able to derive an antibody reacting with granulocytic cells from a mouse immunized with thymocyte glycoproteins.

Samples of human marrow stained by indirect immunofluorescence with anti-HLe-1 were analysed and sorted on a FACS-1. Figure 1a shows the cell sorter profile obtained from such an experiment. Three major populations are separable by combinations of light scattering (cell size) and fluorescence intensity. Table 1 shows the cytological characteristics of the three sorted fractions. The unstained cells (fraction 1) are the recognizable precursors of the erythroid lineage. Fraction 2 contains the cells of the granulocytic lineage and fraction 3 contains mainly small and medium lymphocytes whose staining by indirect fluorescence is similar to that of peripheral blood lymphocytes. Table 1 also gives the colony-forming assay data

Table 1 Differential counts and colony forming cells (CFU-GM) of bone marrow fractions

	Unseparated	Fraction 1	Fraction 2	Fraction 3
Erythrocyte precursors %	13	92	4	0
Blasts and promyelocytes %	0	0	2	0
Myelocytes %	27	4	29	1
Metamyelocytes %	44	3	61	2
Polymorphs %	1	0	3	2
Monocytes %	5	0	0	6
Lymphocytes %	10	1	1	89
CFU-GM per 10 ⁵ cells	39	1.2	89.2	0.6
% Of total nucleated cells	100	17	52	10
% Of total CFU-GM	100	1	117	1

Bone marrow fractions were cytocentrifuged and stained by May-Grünwald Giemsa for differential counting. At least 200 cells were counted in each fraction. Granulocytic and monocytic colonies were grown in semi-solid agar bilayers containing α medium and 25% pre-screened fetal calf serum (FCS)¹. The feeder layer contained 20% placental conditioned medium as a source of colony stimulating activity¹³. The cultures were incubated for 14 days at 37 °C in 5% CO₂ in humidified air and cell aggregates greater than 40 cells were scored as colonies. Values given are the means of three replicate cultures. This experiment has been repeated many times and the above is a representative sample of the results.

Table 2 Effect of TG-1 and complement on differential count and CFU-GM

	F/H marrow	TG-1 + C
Promyelocytes %	1	0
Myelocytes %	10	1
Metamyelocytes %	20	0
Granulocytes %	5.5	0
Lymphocytes %	13	23
Plasma cells %	1	1.5
Monocytes %	5	10*
Erythroid precursors %	35.5	64.5
CFU-GM per 10 ⁵ cells	80	142

* Many of these cells with cartwheel nuclei and ragged cytoplasm are probably degenerating myelocytes and metamyelocytes damaged by the antibody and complement.

(CFU-GM) for the various fractions and control unsorted marrow. The results show unequivocally that the CFU-GM are in fraction 2, but as this fraction contains approximately 50% of the cells isolated from bone marrow by Ficoll-Hypaque separation, this does not represent a significant enrichment of progenitor activity. However, this result does show that the granulocytic/monocytic progenitors appear on FACS analysis in a zone of cells labelled only weakly with anti-HLe-1 in which there are very few lymphocytes. It was therefore possible to identify the correct zone on the cell sorter profile for isolation of the progenitor cells after removing most granulocytic series cells with anti-myeloid antibody and complement.

In a second series of experiments, therefore, bone marrow cells obtained by Ficoll-Hypaque isolation were treated with TG-1 antibody and complement before staining with anti-HLe-1. The TG-1 antibody kills a significant proportion of bone marrow cells of the granulocytic lineage but spares the CFU-GM (Table 2). The cell sorter profile obtained after this sequential treatment is shown in Fig. 1b. Although the erythroid and lymphocyte fractions (fractions 1 and 3) are present, the granulocytic fraction (fraction 2) has been largely removed by the TG-1 treatment. It is still possible, however, to collect the

surviving progenitor cells which fall in the band of cells staining weakly with anti-HLe-1. In the experiment detailed in Fig. 1 and Table 3, two different size fractions of this weakly staining population were collected and these represent somewhat different, though overlapping, progenitor cell populations. The majority of the CFU-GM and the more mature GM clusters and CFU-E are found in the large cell fraction. Both fractions, however, contain large numbers of BFU-E, and as BFU-E are thought to be more primitive than CFU-E or CFU-GM¹¹, it is probable that the smaller cell fraction includes the pluripotential stem cells.

Both fractions contain significant numbers of terminal deoxynucleotidyl transferase (TdT)-positive cells (from 3–15%

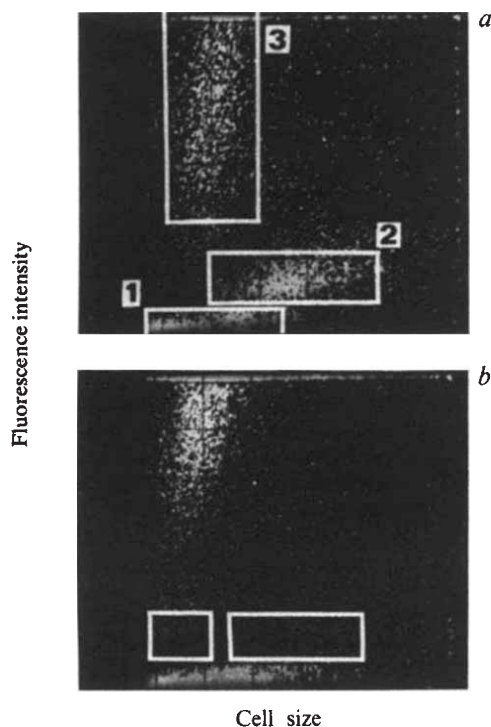


Fig. 1 Bone marrow aspirates were obtained from normal volunteers. The aspirated cells were separated on Ficoll-Hypaque to remove red cells and mature granulocytes and washed in HEPES-buffered RPMI-1640 medium with 5% added fetal calf serum (RPMI-FCS). For analysis or cell sorting, cells were incubated with anti-HLe-1 culture supernatant for 30 min on ice, washed twice with RPMI-FCS and then incubated (30 min on ice) with immunoabsorbent-purified fluorescein-conjugated F(ab')₂ sheep anti-mouse immunoglobulin antiserum (100 µg ml⁻¹ in RPMI-FCS), followed by a further two washes. In some experiments both first and second layers were diluted in 10% normal sheep serum to compete for Fc receptors, a procedure which very effectively prevents Fc binding of mouse immunoglobulin by peripheral blood monocytes (P.C.L.B., unpublished observations). This did not significantly change the FACS profile. For removal of mature cells of the granulocytic lineage, aliquots of bone marrow cells were incubated with TG-1 culture supernatant (2 × 10⁷ bone marrow cells per ml) on ice, washed once and incubated at 37 °C for 45 min with undiluted autologous serum as a source of complement (4 × 10⁷ cells per ml). Surviving cells were washed three times by centrifugation through FCS. This procedure killed approximately 50% of the nucleated cells in the Ficoll-Hypaque-isolated bone marrow. Samples were examined for fluorescence and light scattering on a FACS-1. A typical profile of normal bone marrow stained with anti-HLe-1 is illustrated in *a* and windows for collection of unstained erythroid (1), weakly stained myeloid (2) and brightly stained lymphoid fractions (3) are also shown. *b* Shows the effect of treatment of the marrow with TG-1 and complement before staining with anti-HLe-1. The granulocytic fraction is eliminated but cells can still be collected in the windows illustrated.

Table 3 Differential counts and colony forming data of antibody + complement treated and sorted marrow

	F/H marrow	Fraction 1 (small dim)	Fraction 2 (large dim)
Proerythroblasts %	1	5	14
Normoblasts %	16	6	25
Lymphocytes %	25	37	3
Monocytes %	2	1	0
Plasma cells %	1	1	19
Progenitor cells %	1	47	36
Maturing granulocytic cells %	54	3	3
Clusters-GM } per 10 ⁵ cells	169	1,100	8,450
CFU-GM }	56	700	2,800
CFU-E }	292	3,780	17,130
BFU-E }	34	3,333	2,777

Granulocytic/monocytic clusters and colonies were grown as detailed for Table 1. Clusters are smaller cell aggregates (<40 cells) scored at day 7. Erythroid colonies and bursts were grown in 1.8% methylcellulose containing supplemented α medium, 30% pre-screened FCS and 1% deionized bovine serum albumin¹⁴. Erythroid colonies were grown in the presence of 1 unit ml⁻¹ of human urinary erythropoietin, and were scored at day 7 as benzidine-positive aggregates greater than eight cells. Erythroid bursts were grown in the presence of 3 units ml⁻¹ of erythropoietin and scored at day 14 as haemoglobin-containing aggregates greater than 64 cells. As BFU-E require addition of feeder cells¹⁵, 5 × 10⁴ Ficoll-Hypaque-separated cells were added to each dish for the assays of fractions 1 and 2. The numbers are therefore derived by subtracting the number of bursts produced by 5 × 10⁴ Ficoll-Hypaque cells from the total in each dish. Similar enrichment of erythroid progenitors has been obtained in two other experiments and of granulocytic monocytic progenitors in four. Progenitor cells are mononuclear cells with a high nuclear/cytoplasmic ratio, immature chromatin, with frequent nucleoli and basophilic cytoplasm. No attempt has been made to classify these as myeloblasts, lymphoblasts or undifferentiated blasts.

in different experiments) whereas other sorted fractions do not. The TdT-positive cells in bone marrow are largely Ia (HLA-D) positive and are thought to be lymphoid progenitor cells¹² so that it seems probable that all the bone marrow progenitor cells are included in the weakly HLe-1 stained fraction.

In these experiments we have isolated a small fraction of bone marrow (2% of nucleated cells) which contains all the myeloid and erythroid as well as presumptive lymphoid progenitors. The isolation of such a pure population of progenitors should allow detailed investigation of the regulation of progenitors by other cells, will allow a more exact cytological and antigenic phenotype to be assigned to the progenitor cells and may allow identification of pluripotential stem cells. The study also illustrates the usefulness of combinations of monoclonal antibodies in studies of normal differentiation.

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Mitotic EBNA-positive lymphocytes in peripheral blood during infectious mononucleosis

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Infectious mononucleosis (IM) is usually a benign lymphoproliferative disease caused by Epstein-Barr virus (EBV)¹. Although EBV induces a state of continuous proliferation in infected B lymphocytes *in vitro*^{2,3}, the most prominent lymphoproliferation during IM is of activated, or atypical, T lymphocytes presumably responding to the virus or virus-infected cells⁴. However, EBV genome-carrying cells are known to be circulating during IM, as cultured peripheral blood leukocytes from patients with the disease give rise to continuous lymphoblastoid cell lines, each cell of which contains the EBV genome and expresses the EBV determined nuclear antigen (EBNA)^{5,6}. The proposal that EBV-infected cells in IM blood are not endowed with enhanced growth potential but are merely latently infected⁷ is supported by demonstrations that cells infected *in vivo* enter a viral replicative cycle when placed *in vitro* and that most cell lines derived from cultured lymphocytes of IM patients are infected by virus released *in vitro*⁸. However the cells could also be capable of proliferation *in vivo*, since virus production and transformation are not mutually exclusive properties of EBV-transformed cells⁹. Recently, EBNA has been detected in a very small fraction of peripheral blood lymphocytes of IM patients after T cells were first removed^{10,11} and this has been interpreted to indicate that cell transformation occurs *in vivo* during IM. The isolation of colonies of EBNA-positive cells from IM blood leukocytes cultures in soft agar suggests that at least some infected cells are capable of direct outgrowth into transformed cells¹². We report here direct evidence that circulating EBV-infected cells exhibit increased growth properties during IM.

As EBNA is expressed by EBV genome-carrying lymphocytes in IM blood, and this antigen is physically associated with metaphase chromosomes in dividing transformed cells⁶, we initially determined whether EBNA-positive cells were capable of entering the mitotic cycle after being placed in culture. When cells were incubated for 18 h after separation and then treated

Table 1 Identification of EBNA-positive metaphases in T-cell depleted lymphocytes from patients with IM

Patient	Days of illness	Time (h) cells* in culture	% EBNA	% Of EBNA-positive cells† in mitosis
1	6	18	6.5	5
2	6	18	18	1.0
3	7	2	10	0.5
		18	10	3
4	7	2	12	0.5

Mononuclear leukocytes were isolated on Ficoll-Hypaque gradients¹⁶ from heparinized blood of patients acutely ill with IM. Adherent cells were removed by incubating washed leukocytes in plastic tissue culture flasks for 1 h at 37°C. A population of cells depleted of T lymphocytes and enriched for B lymphocytes was then isolated by separating cells which formed rosettes with neuraminidase-treated sheep erythrocytes (E_N) from non-rosetting cells on a second Ficoll-Hypaque gradient as previously described³. The non-T-cell fraction contained less than 4% E_N rosette-forming cells. 0.5–1 × 10⁶ cells in 0.4 ml RPMI with 20% fetal calf serum were treated for 2 h at 37°C with 0.1 µg ml⁻¹ of colcemid, either immediately following cell separation, or after the separated cells had been cultured overnight (~18 h) at 37°C. Colcemid-treated cells were swollen in 4 volumes of 1% sodium citrate containing 1 mM MgCl₂ and CaCl₂ for 10 min at 37°C. Cell smears were then made on a cytocentrifuge, air dried, fixed for 5 min in acetone/methanol (2:1) and stored at -70°C. EBNA-positive cells in the smears were identified by a modification of the anti-complement immunofluorescence test of Reedman and Klein⁶. Gelatin-veronal buffer (GVB)¹⁷ was used as diluent for sera and for washing slides. Smears were incubated with a human serum containing antibodies to EBNA (RM) or a serum lacking EBV antibodies (LH) which had been diluted 1:8 and heat inactivated. After 30 min at 37°C, the sera were aspirated and the smears were immediately covered with a 1:10 dilution of human EBNA-negative serum as a source of complement. The slides were washed three times in GVB and stained for 30 min at 37°C with a 1:30 dilution of rhodamine-conjugated goat anti-human C3 antibodies (Atlantic Antibodies). After three washes in GVB, the slides were mounted in glycerol-PBS, pH 9.5, and examined in a Zeiss Universal fluorescence microscope equipped with a Ploem type incident illuminator.

*After cell separation completed.

†100–300 EBNA-positive cells counted.

with colcemid, as many as 5% of the EBNA-positive cells were in mitosis (Table 1, Fig. 1a). To identify cells which had already entered the mitotic cycle *in vivo* at the time the blood was drawn, cells were treated with colcemid immediately after cell separation was completed. In two patients (Table 1), a small fraction (0.5%) of the EBNA-positive cells were in mitosis (Fig. 1b). Cells from one of the patients were examined again for EBNA-positive mitoses after 18 h in culture when there was a six-fold increase in the proportion of EBNA-positive cells arrested in metaphase.

We detected EBNA in up to 18% of B-cell-enriched IM blood lymphocytes, although other investigators have identified these cells in only 0.1–2% of cells in similar populations^{10,11}. However, our patients had been symptomatic for 7 days or less whereas the patients studied in earlier reports were usually in their second or third week of illness. We found that the number of EBNA-positive cells decreased rapidly during the second week of illness to levels comparable to those in earlier reports (data not shown). The low numbers of EBNA-positive cells late in disease precluded the detection of mitotic EBNA-positive cells in these specimens.

Rickinson *et al.*⁸ have shown that EBV-neutralizing antibody drastically reduces the outgrowth of transformed cell lines from cultured lymphocytes of IM patients. The antibody presumably acts to neutralize virus released from cells which had been infected *in vivo* but which entered a productive viral cycle *in vitro*. To exclude the possibility that the dividing antigen-positive cells that we detected after 18 h in culture had not been infected by this two-step mechanism, we determined when virus release occurs. We incubated a patient's cells in medium containing EBV-neutralizing antibody at various times after the beginning of culture. As shown in Table 2, EBV-neutralizing antibody did not reduce the frequency of transformation in a quantitative culture assay if it was present only during the first 24 or 48 h. Antibody did reduce the rate of transformation markedly if it was maintained in the cultures for the duration of

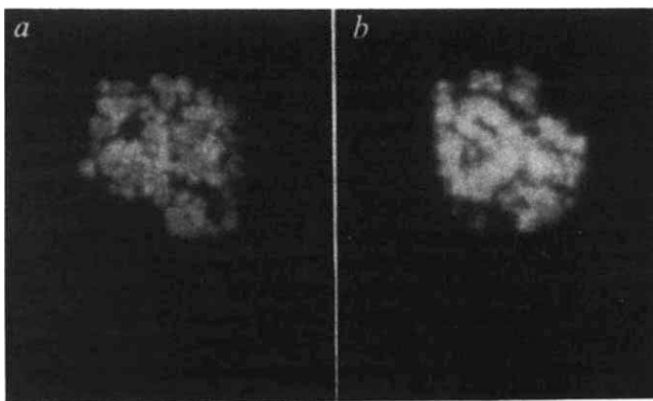


Fig. 1 a, EBNA-positive cell in mitosis. T-cell-depleted lymphocytes exposed to colcemid for 2 h after being cultured for 18 h. ×630. b, EBNA-positive mitotic cell after 2 h exposure to colcemid beginning immediately after cell separation completed. ×630.

the experiment or during the second and third day of culture. Thus, virus production and release do not occur during the first 2 days of culture. Even if normal cells could be infected at the time cells were placed *in vitro*, proliferation does not begin immediately. As shown in Table 3, cell division in normal B lymphocytes inoculated with EBV *in vitro* does not begin until 34–46 h after exposure to virus. Thus, our results show that a proportion of cells infected by EBV *in vivo* are capable of cell division after being placed in culture and that some of the antigen-positive cells have already traversed the cell cycle while still in the peripheral blood.

The capacity of the dividing EBNA-positive cells to grow into continuous cell lines is unknown. They may be the same as the cells which form EBNA-positive colonies in soft agar¹². However, the ability to grow directly into cell lines is not a prerequisite for establishing that transformation occurs *in vivo*. We recently studied a 4-year-old girl who developed a poorly differentiated lymphoplasmacytic malignancy during IM. The tumour cells and the majority of peripheral blood leukocytes contained both EBNA and cytoplasmic immunoglobulins of all heavy and light chain classes¹³. Although in this patient there was unequivocal evidence of viral transformation *in vivo*, her cells either entered a lytic viral cycle or failed to grow in culture; only a few ultimately formed cell lines in soft agar.

During IM, the host immune response to EBV infection, which is usually already evident at the time of diagnosis^{14,15}, is probably responsible for restricting EBV-induced proliferation to the relatively low levels that we observed *in vivo*. Were it possible to study IM patients before symptoms develop, much higher numbers of dividing EBNA-positive cells might be seen.

Table 2 Time of effectiveness of EBV-neutralizing antibody in reducing the transformation frequency of cultured B-lymphocytes from IM blood

Time period antibody present in cultures				Transformation frequency*
0–24 h	24–48 h	48–72 h	8 weeks	
–	–	–	–	23/24
+	+	+	+	10/24
+	–	–	–	21/42
+	+	–	–	24/24
+	+	+	–	15/24
–	+	+	+	7/24

T-cell-depleted lymphocytes were isolated from a patient with IM on the 6th day of illness as described in Table 1. Replicate cultures of cells containing 5×10^5 cells were incubated at 37 °C for 24-h periods in medium RPMI 1640 supplemented with 15% fetal calf serum and 5% human serum from either an EBV-seropositive (EBV neutralization titre = 1:80) or seronegative individual. Each group of cells was washed three times in RPMI after 24, 48 and 72 h in culture and then resuspended in 0.5 ml of medium with or without antibody. At 72 h, the cells in each treatment group were plated at 10^4 cells per well in microtitre tissue culture plates previously seeded with human placental fibroblast feeder layers as previously described³. Cultures were fed weekly by removing half of the medium and replacing it with medium with and without antibody. Cultures were observed for approximately 8 weeks and the number of wells showing transformation in each treatment group were scored. +, Antibody present; –, antibody absent.

*No. of transformed wells/no. of wells plated.

Table 3 Time course of EBV-induced proliferation in B-lymphocytes inoculated with EBV *in vitro*

Time after inoculation (h)	% EBNA	EBNA-positive mitoses per 1,000 cells
10	7	0
22	44	0
34	51	≤1
46	55	16
58	56	94

Lymphocytes depleted of T cells as in Table 1 were isolated from a normal individual and incubated with concentrated EBV (B95-8 strain; multiplicity of infection = 500 physical particles per cell) or medium at 4 °C for 2 h. After three washes, the cells were resuspended in medium at 1.5×10^6 cells ml⁻¹. Aliquots (0.4 ml) of these cells were cultured in 13 × 100 mm stoppered tubes at 37 °C in an atmosphere of 5% CO₂. Beginning 10 h after the cultures were initiated, infected and uninfected cells were treated with 0.1 µg ml⁻¹ colcemid at 12-h intervals; smears for EBNA and EBNA-positive mitoses were prepared as described in Table 1.

The increase in the proportion of EBNA-positive cells in mitosis 18 h after being placed in culture suggests a release from the influence of an inhibitory environment. Since no mitotic cells were seen in Giemsa-stained slides prepared from 18-h cultures of T-cell-depleted lymphocytes from four normal individuals, it is more likely that the increased mitotic activity is due to the presence of the EBV genome in the cells than to a nonspecific stimulation of the cells resulting from the absence of T cells or other factors. If adequate host control mechanisms fail to develop *in vivo* during IM, as was likely in our fatal case¹³, the EBV-infected cells have the potential for causing a malignant lymphoproliferation.

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Isoelectric focusing of IgG eluted from multiple sclerosis and subacute sclerosing panencephalitis brains

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Elevated IgG distributed in oligoclonal bands is characteristically observed in the cerebrospinal fluid (CSF) of patients with multiple sclerosis (MS) or subacute sclerosing panencephalitis (SSPE)^{1–6}. Similarly, IgG in bands has been detected in neutral saline (NS) and acid eluates of brain material from these two diseases^{7–21}. We have now used isoelectric focusing (IEF) to compare IgG eluted from control brain, three plaques and a white matter pool of an MS brain, and three regions of an SSPE brain. A direct peroxidase-conjugated anti-human IgG staining technique was used to stain IgG exclusively and to visualize the minute amounts of IgG obtained from individual MS plaques. Eluates from individual MS plaques have distinct IgG patterns; in contrast, those from separate SSPE brain areas have essentially identical IgG patterns. The identical IgG patterns in three areas of SSPE brain suggest a common response to the same antigen. The different IgG patterns among MS plaques suggest: (1) variable response to the same 'MS antigen' in each plaque, (2) response to different MS antigens in different plaques, (3) synthesis of 'nonsense' antibodies irrelevant to the pathogenesis of MS in each plaque, or (4) some combination of the above.

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IgG was eluted by modifications of the techniques of Gilden^{21,22} and Weil^{14,15} from: the white matter pool (WMP) and three plaques of an MS patient, three discrete brain areas of an SSPE patient, and a WMP of an individual without neurological disease. Although seven NS washes reduced recoverable IgG to nearly zero in all material, subsequent acid elution recovered significant additional IgG (Table 1). The IgG eluted from these tissues, along with serum and CSF from the SSPE case, was then examined by IEF.

NS and acid eluates from each MS plaque had unique overall patterns of IgG bands on IEF despite many shared bands (Fig. 1). The WMP acid eluate contained all the IgG bands found in the acid eluates from the individual plaques plus additional bands. NS eluates had more anodal and fewer cathodal bands than acid eluates. The difference in pattern between acid and NS eluates could not be accounted for by acid exposure because treatment of NS-eluted IgG with acid did not change the IEF pattern. Analysis of three discrete 0.6–1.2 g white matter regions from a second MS case (age 36 yr, with a 13-yr history of chronic progressive MS) has given comparable differences in banding patterns.

The oligoclonal IgG patterns of the NS eluates of all three SSPE brain regions were identical, and similar to the pattern of the CSF. The serum had several bands, identical with bands in the CSF. Acid eluates of the three SSPE brain regions had virtually identical oligoclonal IgG patterns (Fig. 2). When compared with NS eluates and CSF, acid eluates had an additional major cathodal band and other minor differences. A second case of SSPE (age 14 yr, with an 8-month duration of disease and a history of primary measles at age 4 yr) has given similar results.

NS and acid eluates from control WMP showed indistinct bands very different from the sharp bands observed in MS and

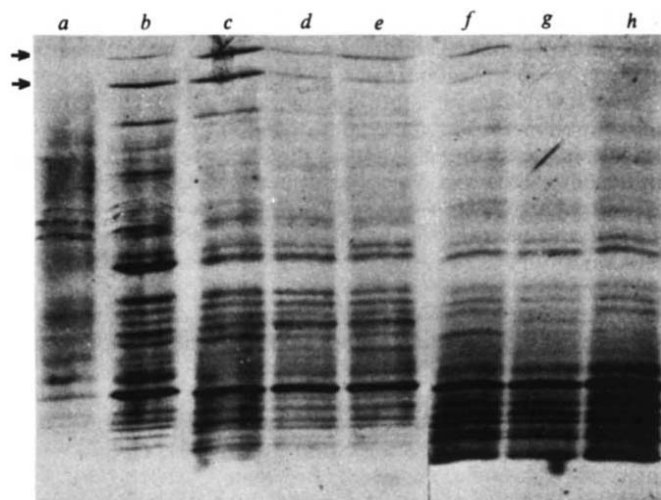


Fig. 2 IEF of SSPE brain material, serum and CSF. *a*, Postmortem serum; *b*, postmortem CSF; *c*, region 1 1st + 2nd NS eluates; *d*, region 2 1st + 2nd NS eluates; *e*, region 3 1st + 2nd NS eluates; *f*, region 1 1st + 2nd acid eluates; *g*, region 2 1st + 2nd acid eluates; *h*, region 3 1st + 2nd acid eluates. IEF as in Fig. 1.

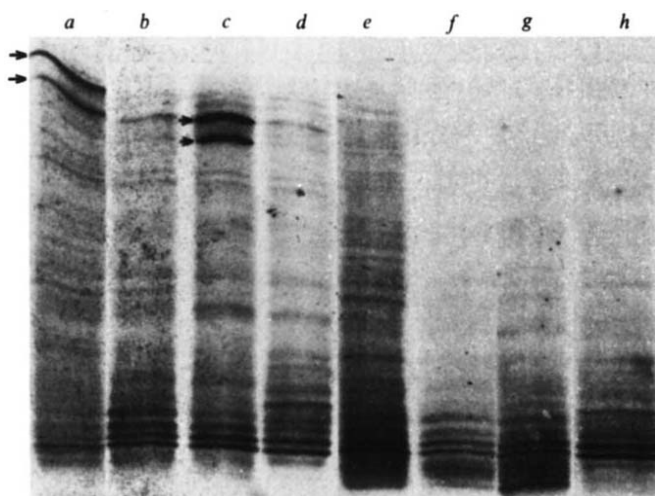


Fig. 1 IEF of MS brain material. *a*, WMP 1st + 2nd NS eluates; *b*, plaque 1 1st + 2nd NS eluates; *c*, plaque 2 1st + 2nd NS eluates; *d*, plaque 3 1st + 2nd NS eluates; *e*, WMP 1st + 2nd acid eluates; *f*, plaque 1 1st + 2nd acid eluates; *g*, plaque 2 1st + 2nd acid eluates; *h*, plaque 3 1st + 2nd acid eluates. The top of the gel is pH 7.0 and the bottom is pH 9.0. Arrows indicate haemoglobin which stains brown with our peroxidase substrate and is present especially in the 1st + 2nd NS eluates. IEF was carried out using LKB Ampholine PAGplate gels, pH 3.5–9.5, on an LKB 2117 Multiphor with an LKB 2103 power supply. The anode solution was 1 M H_3PO_4 ; the cathode solution 1 M NaOH. Samples containing 2 μ g of IgG were applied to the middle of the gel in 5 × 10 mm pieces of filter paper and focused for 2 h at 4 °C and 30 W constant power. Immediately after focusing, gels were fixed for 1 h with trichloroacetic acid (TCA) and sulphosalicylic acid (SSA) (57.5 g TCA and 17.25 g SSA in 150 ml methanol and 350 ml water), and rinsed for 48 h in Tris buffer (0.1 M Tris-HCl, 0.85 M NaCl, pH 7.6). A cellulose acetate strip soaked with undiluted peroxidase-conjugated rabbit anti-human IgG (Dako), 40 μ l per sample lane, was placed on the gel for 1 h at room temperature in a moist environment. Gels were rinsed with Tris buffer in the dark for 96 h, and developed for 1 h in 30 mg% 3,3'-diaminobenzidine tetrahydrochloride (Sigma) and 0.06% hydrogen peroxide (in Tris buffer).

SSPE eluates (Fig. 3). These bands were more readily discerned in the NS than in the acid eluate. Diffusely distributed cathodal IgG was more evident in the acid than in the NS eluate.

IgG obtained after acid treatment of normal brain may be nonspecifically bound (through Fc receptors^{23,24} or charge affinities), or released from fractured plasma cells. The oligoclonal pattern of this IgG, an observation made previously in NS eluates using agar gel electrophoresis (AGE)¹⁹, may reflect sequestration of certain lymphoid cell clones in normal brain.

Acid elution of SSPE brain is thought to dissociate complexes of anti-measles oligoclonal IgG from measles antigen^{14,15,18,20}; NS eluted IgG probably represents antibody in excess of antigen. This view is supported by the observation that most acid- and NS-eluted IgG bands, as well as CSF IgG bands, are absorbable by measles virus^{13,17,18}. All three SSPE brain regions we examined had almost identical banding patterns, suggesting a restricted antibody response to the same antigen in each area. CSF and NS eluates, however, contained several IgG bands not apparent in acid eluates of brain. These bands may not be anti-measles antibodies but may instead be the product of nonspecific clones recruited to the brain. SSPE CSF oligoclonal IgG bands not absorbable with measles virus have been reported^{13,17,18}.

The three MS plaque acid eluates analysed had several bands in common, but each had a unique overall pattern. The acid eluate of MS WMP contained all bands present in the acid eluates of the three plaques plus additional bands, as if patterns from many plaques had been summed. Although some differences in band patterns may be quantitative rather than qualitative, the variation in band pattern from plaque to plaque indicates that different antibody-synthesizing clones are present (or expanded) in different areas of MS brain.

Several explanations for this observation can be advanced. If oligoclonal IgG eluted with acid from MS brain is antibody specifically bound to an MS antigen(s), plasma cell clones producing antibody directed against MS antigen(s) may appear, decline and disappear in different plaques over time. Old, chronic or inactive plaques might then be expected to contain different clones from more recent, active plaques. IgG bands (in serum) are known to change over time in animals chronically stimulated with a well defined antigen^{25,26}. Our failure to find any changes in different regions of the brain in SSPE perhaps argues against this interpretation. Although the SSPE cases studied were less than 1 yr in duration, one would have thought

that this was sufficient (especially considering an 'incubation period' of at least 10 yr from initial measles infection) for changes to have taken place.

Another explanation, which again would require that all acid-eluted antibody is specifically bound, would be that a different antigen is present in each MS plaque and for this reason antibody-synthesizing clones differ in each plaque. This seems improbable; however, a single agent can undergo antigenic shifts leading to different antibody responses over time. Antigenic shifts have been observed with visna virus in infected sheep brains²⁷.

A third possibility is that those bands common to all plaques are specific antibody directed against an MS antigen(s), whereas those bands unique to individual plaques are nonsense antibody irrelevant to the pathogenesis of MS. Such nonsense antibody would be the product of plasma cell clones that become localized in the plaque but are not stimulated by any antigen present there. This irrelevant IgG would be released by acid treatment through breakage of nonspecific bonds or rupture of plasma cells. Antibodies to several viruses, such as measles and rubella, are elevated in MS CSF and are oligoclonal in distribution²⁸. Although these antibodies do not correspond to the CSF oligoclonal IgG bands seen by AGE, they could appear as bands when CSF or brain eluates are analysed by the more sensitive IEF technique. Of relevance is the observation that mice immunized with several antigens and boosted in a lymph node with one, show recruitment to the node not only of plasma cells specific to the stimulating antigen, but also of lymphoid clones directed against the other antigens²⁹. Thus, there is a precedent for the notion that irrelevant plasma cell clones can be recruited in the face of specific antigenic challenge.

Finally, all IgG in MS plaques could be nonspecifically bound, irrelevant to any pathogenic antigen, and represent the product of B-cell clones sequestered randomly during attacks of MS. Bands common to eluates of all plaques would then represent the product of clones constantly maintained by repeated or continuous antigenic exposure whereas bands unique to a plaque would be the product of clones responding to antigens encountered shortly before activation of a plaque by an MS attack.

We believe that resolution of the questions raised by these findings may prove crucial for an understanding of the pathogenesis of MS.

SSPE material was obtained from Dr J. H. Webster. This work was supported by grants from the National Multiple Sclerosis Society (RG 1130-C-16), the Kroc Foundation, the Medical Scientists Training Program (PHS 2 T32 GM07281) to

Table 1 IgG recovered from MS, SSPE and control brain material on neutral saline and acid elution

Material (weight)	IgG recovered at various elution steps (µg)		
	1st+2nd NS	7th NS	1st+2nd acid
MS white matter pool* (6.0 g)	851 (0.142)§	7.7	390 (0.065)§
MS plaque 1* (0.1 g)	57 (0.574)	0	8.9 (0.089)
MS plaque 2* (0.4 g)	113 (0.283)	0.1	18 (0.045)
MS plaque 3* (0.4 g)	131 (0.328)	0.5	20 (0.050)
SSPE cortex region 1† (2.3 g)	1,222 (0.531)	4.1	97 (0.042)
SSPE cortex region 2† (1.8 g)	1,407 (0.782)	0.9	66 (0.037)
SSPE cortex region 3† (2.1 g)	435 (0.207)	1.8	82 (0.039)
Control white matter pool‡ (6.3 g)	641 (0.102)	4.1	41 (0.006)

Sections 8 µm thick were cut on a cryostat and homogenized with 10 strokes of a Dounce homogenizer in a small amount of Dulbecco's phosphate-buffered saline (DPBS) containing 0.02% sodium azide. Each homogenate was rinsed seven times with at least 7 volumes of DPBS containing 0.02% sodium azide at 4 °C, with centrifugations at 100,000g for 1 h at 4 °C. Homogenates were then rinsed twice with similar volumes of 0.1 M glycine-HCl buffer at pH 2.5 (containing 0.1% bovine serum albumin, 0.005 M ϵ -aminocaproic acid and 2.8 µg ml⁻¹ pepstatin), each time for 1 h at 4 °C with end over end shaking, followed by centrifugations at 100,000g for 1 h at 4 °C. Immediately after centrifugation, supernatants from each acid elution were brought to pH 7.0 by addition of 1 M Tris(hydroxymethyl)aminomethane (Sigma). The pooled first plus second neutral saline rinses, the seventh neutral saline rinse and the pooled first plus second neutralized acid eluates were concentrated using vacuum ultrafiltration with 25,000 molecular weight cutoff collodion bags (Schleicher and Schuell) against several changes of a 1:10 dilution of DPBS. IgG was quantified in the concentrated eluates by electroimmunodiffusion (Antibodies Incorporated).

* Periventricular white matter and three brain plaques were dissected at autopsy 13 h after death and stored at -70 °C for 2 yr. The patient was a 51-yr-old female with a 9-yr history of chronic progressive MS.

† Three separate areas of cortical white (with adjoining grey) matter were dissected at autopsy 6 h after death and stored at -70 °C for 1 week. Postmortem CSF and serum were also obtained. The patient was a 17-yr-old male with a 10-month history of SSPE. He had a primary measles infection at age 6 yr.

‡ White matter was dissected at autopsy 20 h after death and stored at -70 °C for 2 months. The patient was a 47-yr-old male with lung carcinoma.

§ Values in parentheses are µg per mg IgG recovered from starting material.

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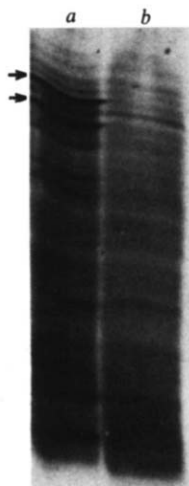


Fig. 3 IEF of control brain material. a, WMP 1st + 2nd NS eluates; b, WMP 1st + 2nd acid eluates. IEF as in Fig. 1.

Limitations in the use of actomyosin threads as model contractile systems

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Recent studies have suggested that actomyosin threads may provide a useful model for studying the properties of contractile systems¹⁻³. The development of highly sensitive positional feedback transducers has enabled the properties of these threads to be measured reproducibly¹. Potential applications include such systems as ventricle, smooth muscle and non-muscle preparations, from which it is difficult to obtain suitable fibres for mechanical studies. In addition, studies with chemically modified myosins may provide new insights into the relationships between the biochemical and mechanical events in the cross-bridge cycle. However, there are indications that the mechanical properties of actomyosin threads differ from those of intact fibres in several important respects. For example, contraction velocity is proportional to isometric tension in threads², but is independent of filament density in intact fibres⁴. We have now determined the force-velocity characteristics of actomyosin threads prepared from muscles with known differences in their physiological contraction velocities. No direct relationships could be found between the velocity characteristics of the threads and those of intact muscle. We conclude that the measured velocities of threads reflect properties of the actomyosins other than cross-bridge cycling times, thus severely limiting the usefulness of this technique for comparative purposes.

Natural actomyosins were isolated from the ventricle and back (fast skeletal) muscles of rabbit, and from the red (slow) and white (fast) myotomal muscles of the dogfish *Scyliorhinus canicula*⁵. Purified actin⁶, myosin⁷ and tropomyosin-troponins⁶ were also prepared from rabbit back muscle. Threads were formed using the method described by Crooks and Cooke¹, involving the extrusion of actomyosin at high ionic strength from a syringe needle moving through a solution of low ionic strength. Isometric contraction was initiated by the addition of ATP or CaCl₂ (Fig. 1). Calcium sensitivity (as defined by Lehman and Szent-Györgyi⁵) in the threads was difficult to achieve, even though ATPase activities of the actomyosins were typically >80% calcium sensitive and gel electrophoresis showed the presence of calcium regulatory proteins in the threads. Isotonic contraction velocities were measured in maximally activated threads by release against a series of electronically present loads. The force-velocity curves obtained in this manner are shown in Figs 2 and 3, and the unloaded contraction velocities (V_{max}) and maximal tensions (P_0) are listed in Table 1, together with ATPase activities for the actomyosins.

The data reveal several unphysiological characteristics. First, the velocities are all less than 0.012 lengths s⁻¹, two to three orders of magnitude lower than those of intact muscle fibres. For example, V_{max} values for *Tilapia* adductor operculi white and red fibres are 2.6 and 1.5 lengths s⁻¹ at 18°C (ref. 8); Lännergren reports values of 6.3 and 1.1 lengths s⁻¹ for *Xenopus* fast and slow fibres (22.5°C), and Close gives values of 14.5 and 6.3 lengths s⁻¹ for whole extensor digitorum longus and soleus muscles of the rat (35°C)¹⁰.

Second, the relative velocities of the different thread types do not reflect those of the corresponding muscles (Figs 2, 3, Table 1). Barany¹¹ has shown a positive correlation between the actomyosin ATPase activity and the contraction velocity of a muscle. However, the velocities of the threads from both animals show a complete reversal of the results predicted from both the ATPase levels and the studies noted above on single fibres

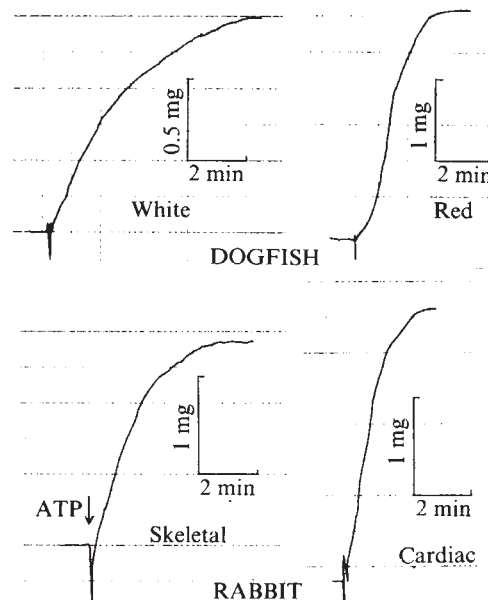


Fig. 1 Development of isometric tension with time in actomyosin threads activated with ATP. Incubation medium contained 50 mM imidazole, 40 mM KCl, 5 mM EGTA, 5 mM CaCl₂, 3 mM MgCl₂, 0.5 mM Na₄P₂O₇ and ATP added to a final concentration of 500 μ M. An ATP regenerating system was also present (10 mM creatine phosphate, creatine phosphokinase at >20 units ml⁻¹). Protein purity was examined by SDS-polyacrylamide gel electrophoresis and electron microscopy. Natural actomyosins were used the same day as preparation, purified component proteins directly after purification. Protein concentrations varied from 50 to 65 mg ml⁻¹ (determined by a microbiuret method¹⁸). Tension and force-velocity parameters were measured on a sensitive tensiometer similar in design to that described by Crooks and Cooke¹ (sensitivity in isotonic: 100 mV per 70 μ m; in isometric: 135 mV mg⁻¹; drift 0.02 mg h⁻¹; compliance 0.1 μ m mg⁻¹). Threads 5 mm long and 240 μ m in diameter were attached to the tensiometer using a Plexiglass-acetone glue.

and whole muscles from different muscle types. It therefore seems that the measured contraction velocities of actomyosin threads reflect factors other than cross-bridge cycling times. The most important of these is probably related to the geometry and packing of filaments within the threads. Threads lack the polarity and native organization of myofilaments found in intact muscle¹². As noted above, Cooke and Franks² found velocity to be proportional to isometric tension (which is determined by protein concentration) in threads, whereas it is independent of myofibrillar density in intact muscle⁴.

Ultrastructural studies by D'Haese and Komnick¹² have shown that contraction of actomyosin threads results in a condensation of the parallel filament system, in cross-section, and increased overlap of filaments in longitudinal section, suggesting that contraction is due to a sliding filament mechanism. These studies also indicate that the conditions of myosin filament formation may determine the properties of the threads. For example, threads prepared by rapid precipitation contain actin filaments decorated with myosin molecules and oligomeric myosin bridges. During isometric contraction, myosin filaments form and, with actin filaments, align themselves along the long axis of the thread. Dialysis of the actomyosin solution before thread extrusion, to give slower myosin precipitation, produces threads with both actin and myosin filaments in the relaxed state. The initial unloaded contraction velocities of these threads is over four times greater than those of rapidly precipitated threads.

Other studies have shown that filament formation by purified myosin depends to a large extent on the conditions of formation

Table 1 Unloaded contraction velocity, maximal tension and ATPase activity for actomyosins

Actomyosin source	Mean $V_{\max}$ ($\text{L s}^{-1} \times 10^2$)	Mean P_0 ($\text{mN cm}^{-2} \times 10^3$)	Actomyosin ATPase activity ($\mu\text{m Pi mg}^{-1} \text{min}^{-1}$)
Rabbit			
Skeletal	0.53 ± 0.05	59.8 ± 17.2	0.51
Cardiac	1.16 ± 0.14	67.1 ± 16.8	0.07
Dogfish			
White	0.18 ± 0.06	26.0 ± 9.7	0.55
Red	0.39 ± 0.12	57.0 ± 4.1	0.18

L s^{-1} = thread lengths s^{-1} ; errors indicate ± 1 s.d. ATPase activities were determined in an incubation medium of 50 mM imidazole, 40 mM KCl, 0.1 mM CaCl_2 , 3 mM MgCl_2 , 2.5 mM ATP and protein concentration 0.1–0.5 mg ml^{-1} ; rabbit activities were measured at 37 °C, pH 7.0, dogfish activities at 15 °C, pH 7.4.

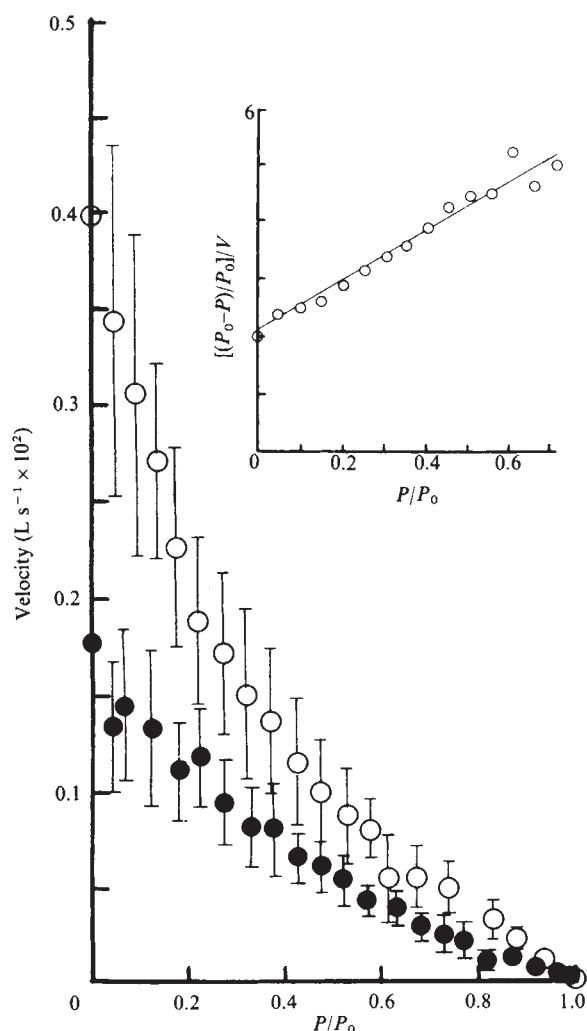


Fig. 2 Force-velocity relationships for dogfish threads at 15 °C, pH 7.4. $\circ$, Red muscle; $\bullet$, white muscle. Velocities are in thread lengths s^{-1} ; P/P_0 = force applied/maximal isometric tension. Error bars indicate ± 1 s.d. The data are derived from 6 threads of red actomyosin from 3 animals, and 14 threads of white actomyosin from 6 animals. The inset shows a typical curve for red actomyosin plotted according to the linear form of Hill's equation¹⁹. Points below 0.7 P_0 lie on a straight line. A similar fit has been described for intact single fibres⁹.

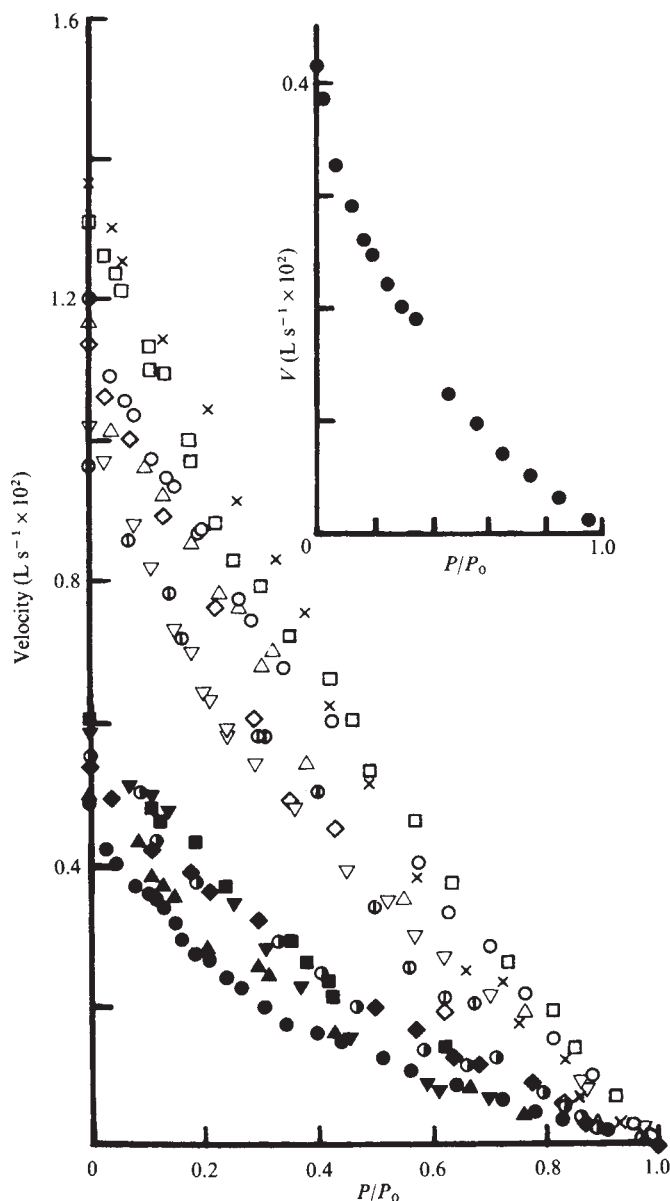


Fig. 3 Force-velocity relationships for rabbit threads at 37 °C, pH 7.0. Open symbols represent seven individual threads of cardiac muscle from three animals; solid symbols present six threads of fast skeletal muscle from three animals. The inset shows a typical force-velocity curve for a thread prepared from purified actin, myosin and regulatory proteins of skeletal muscle, demonstrating its similarity to threads of skeletal natural actomyosin.

and the age of the myosin. The length, diameter and shape of synthetic myosin filaments are affected by pH (refs 13, 14), ionic strength¹⁴ and the concentrations of ATP, PO_4^{3-} , Mg^{2+} and Ca^{2+} (ref. 15). Specific concentrations of ATP and Mg^{2+} are needed for the formation of filaments with physiological diameters, tapered ends and a central zone of polarity reversal¹⁵. Storage of myosin in the cold results in the loss of the ability to form these 'physiological' filaments¹⁶. During storage there is a loss of the LC2 light chain; removal of the LC2 chain from fresh myosin by treatment with dithionitrobenzene (DTNB) mimics this ageing process.

Structural differences among the myosins of fast, slow and cardiac muscles have been well documented¹⁷, and it is possible that these myosins will differ in their abilities to form filaments in the conditions required to prepare and activate threads.

Our results, in conjunction with the above studies, suggest that filament formation, geometry and packing, and not

differences in cross-bridge cycling rates, largely determine the observed properties of actomyosin threads. Thus, any physical or chemical manipulation (such as protein concentration, storage time, ion concentrations, DTNB treatment) of the actomyosin that alters its ability to form filaments is likely to result in secondary, unphysiological changes in the contractile properties of threads.

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Nuclear accumulation of epidermal growth factor in cultured rat pituitary cells

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Epidermal growth factor (EGF) is a mitogen for epidermal cells *in vivo*^{1,2} and for a wide variety of cells in culture^{3–5}. Recently, we and others have reported that EGF can also regulate the cellular levels of various hormones⁶ and fibronectin⁷ at concentrations which only minimally influence cell division. In addition, EGF treatment of GH₃ cells affects chromatin structure such that isolated nuclei from treated cells have an increased capacity to bind bacterial RNA polymerase in initiation site complexes⁸. Thus, the data suggest that various nuclear functions are modulated by EGF in GH₃ cells despite its failure to affect DNA synthesis or cell proliferation. Recently, Yanker and Shooter⁹ have reported on the nuclear accumulation of nerve growth factor (NGF) in PC12 cells in which NGF does not promote cell division but does influence RNA and protein synthesis while inducing overt differentiation (neurite outgrowth). The similarities between the two systems and the various theories regarding the mechanism by which mitogens exert their growth-promoting and other effects^{9,10} led us to investigate whether an interaction between EGF and the cell nucleus can be demonstrated after surface binding and internalization of EGF in GH₃ cells. We report here that when its lysosomal degradation is inhibited by chloroquine, EGF accumulates in the nucleus.

Previous studies with GH₃ and other cell types have demonstrated that the cell-bound ¹²⁵I-EGF is rapidly degraded ($t_{1/2}$ = 20 min) to mono-¹²⁵I-iodotyrosine by lysosomal proteases following endocytotic internalization^{6,11,12}. Due to this process, the intracellular amount of intact EGF reaches, within 1–2 h of exposure to EGF, a steady-state concentration which is much lower than the actual amount of internalized EGF and which later decreases even more due to a loss (down regulation) of the cell-surface receptors for the factor. Furthermore, if translocation of EGF into the nucleus does occur, this might require a time longer than the mean half life of the internalized EGF molecules, thereby even further reducing the possibility of detecting significant quantities of nuclear-bound ¹²⁵I-EGF. Therefore, our approach in probing for nuclear accumulation of EGF in GH₃ cells was to use the lysosomal inhibitor chloroquine, which has been shown to inhibit, in this and other cell types, the degradation of EGF and thus allows the intracellular accumulation of EGF^{4,6}.

In the following experiments, cells were incubated with ¹²⁵I-EGF in the absence and presence of chloroquine, lysed with buffer containing 0.5% NP40, and tested for the amount of radioactivity associated with the nuclear pellet and cell lysate. As shown in Fig. 1a, nuclei isolated from detergent-lysed cells which were exposed to 12.5 ng ml⁻¹ ¹²⁵I-EGF for 18 h contained about 1% of the cell-associated label. In contrast, when cells were incubated with EGF in the presence of increasing concentrations of chloroquine, the amount of nuclear-associated radioactivity increased and reached a plateau of about 14% of the cell-bound counts at a concentration of 75 μ M chloroquine. In these conditions, the total amount of cell-associated ¹²⁵I-EGF (bound plus internalized) was increased sixfold. As shown in Fig. 1b, the nuclear accumulation of EGF in chloroquine-treated (75 μ M) cells lags significantly behind the rate of internalization of the growth factor. Thus, after a 4-h incubation with EGF, only about 2% of the internalized EGF (measured in the NP40 lysate) was associated with the isolated nuclei, whereas a 24-h incubation resulted in a nuclear accumulation of as much as 12% of the internalized EGF. Such a difference in the kinetics of cytoplasmic accumulation and nuclear binding also suggests that the observed nuclear accumulation of EGF is not due to contamination of the nuclei by membrane- or cytoplasm-bound EGF. This was further documented by electron microscopy of nuclei prepared from control and chloroquine-treated cells (Fig. 1c, d). In both cases the nuclei were free of the lysosomal vesicles known to accumulate in chloroquine-treated cells. Nuclei were also tested for lysosomal and plasma membrane contamination by assaying the biochemical marker enzymes acid phosphatase and (Na⁺ + K⁺)ATPase respectively. Acid phosphatase activity in the nuclei measured by the method of Brandenberger *et al.*¹³ was less than 0.5% of the total cellular activity in both control and chloroquine-treated cells. Nuclear (Na⁺ + K⁺)ATPase measured according to Jackson¹⁴ was less than 0.3% of the total cellular activity and similarly was not influenced by chloroquine treatment of the cells.

The possibility that the observed nuclear binding occurred not within the cell but upon lysis in detergent buffer was next examined. Cells were incubated for 18 h with 75 μ M chloroquine in the presence or absence of 2 ng ml⁻¹ ¹²⁵I-EGF. After washing the cultures were lysed with the NP40 buffer containing no EGF, 10 μ g ml⁻¹ unlabelled EGF (in the case of prelabelled cells) or 2 ng ml⁻¹ ¹²⁵I-EGF (in the case of unlabelled cells). Nuclei were then isolated as in Fig. 1 and the bound EGF determined. Nuclei isolated from 10⁶ cells prelabelled for 18 h with ¹²⁵I-EGF contained 5.1 fmol of ¹²⁵I-EGF while those prelabelled and lysed in buffer containing 10 μ g ml⁻¹ unlabelled EGF contained 4.98 fmol ¹²⁵I-EGF. However, nuclei isolated with lysis buffer containing ¹²⁵I-EGF contained only background levels of radioactivity. This suggests that the observed nuclear binding occurs before lysis of the cell. Furthermore, incubation of cells with ¹²⁵I-EGF and chloroquine at 4 °C rather than 37 °C was not associated with cytoplasmic or nuclear

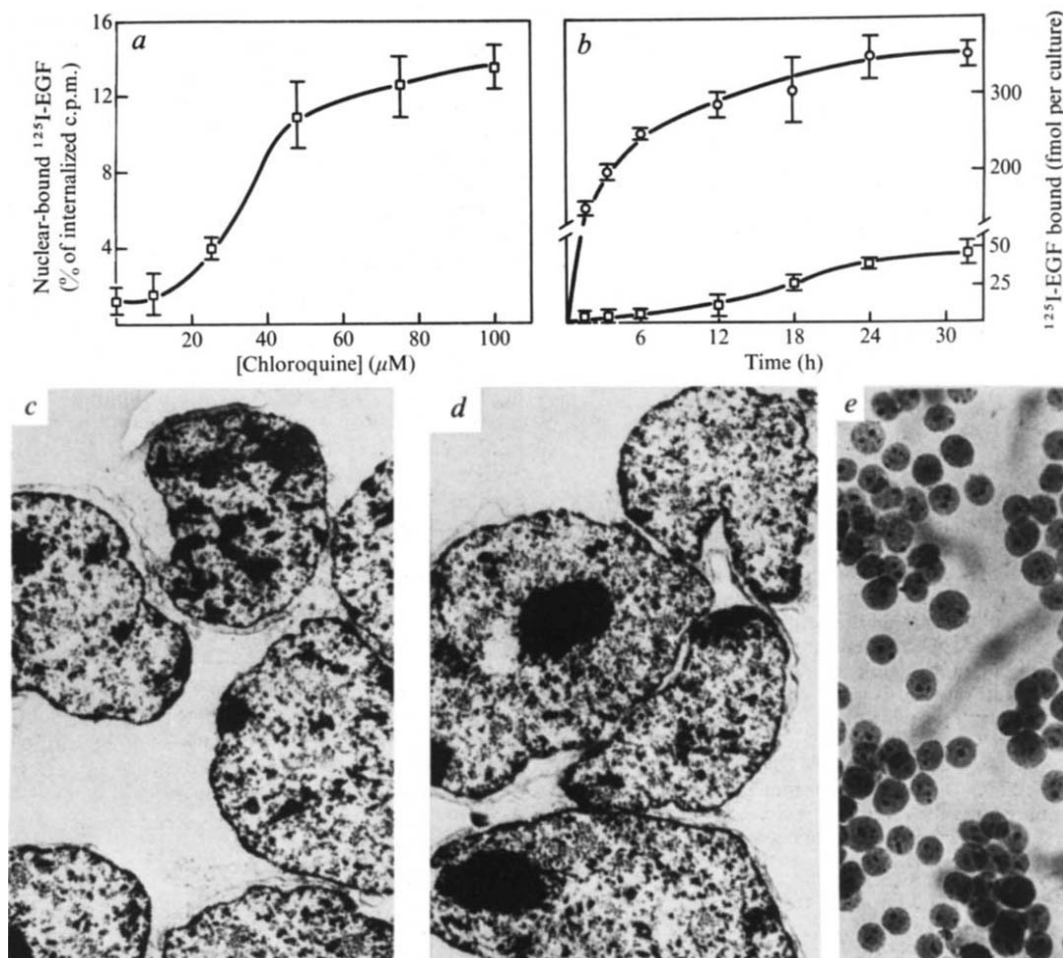


Fig. 1 Nuclear accumulation of ^{125}I -EGF in GH₃ cells. *a*, Effect of chloroquine concentration on the per cent of total cell-associated ^{125}I -EGF found in the nuclear fraction after 18 h. *b*, Kinetics of ^{125}I -EGF accumulation in GH₃ cell lysates and nuclei. 5×10^6 GH₃ cells, maintained as previously described⁶, were seeded into 35-mm culture dishes. After 24 h the medium was replaced by 1.0 ml of medium containing 0.5% fetal calf serum and the indicated chloroquine concentrations. One hour later 12.5 ng ml^{-1} ^{125}I -EGF (purified and iodinated as described⁴; specific activity, $460,000 \text{ c.p.m. ng}^{-1}$), was added in the presence and absence of $2.5 \text{ } \mu\text{g ml}^{-1}$ unlabelled EGF. After incubation at 37°C cultures were washed 10 times with phosphate-buffered saline containing 1 mg ml^{-1} BSA and lysed in buffer containing 0.5% NP40 (ref. 6). The initial supernatant after removal of the nuclei ($1,000\text{g}$, 5 min) was designated the lysate fraction (open circles) and the nuclei were purified further as described⁶ before counting of radioactivity (open squares). The mean and range of triplicate determinations are shown for each experiment. Electron microscopy of nuclear pellets from control (*c*) and chloroquine-treated (*d*) cells. Pellets were fixed on ice in 4% glutaraldehyde, 3.6% formaldehyde, 0.2 M cacodylate buffer (pH 7.2). Pellets were postfixed in 1% osmium tetroxide, 0.1 M Na cacodylate (pH 7.2), dehydrated in acetone and embedded in epon 812. Sections were stained with uranylacetate and lead citrate and photographed at an initial magnification of $4,500\times$. *e*, Phase contrast micrograph of nuclei from chloroquine-treated cells. Nuclei were stained with 1% trypan blue and photographed at an initial magnification of $200\times$.

accumulation of ^{125}I -EGF, demonstrating that the nuclear uptake is dependent on internalization of the growth factor.

Having found conditions for experimentally detecting nuclear-associated ^{125}I -EGF, we next asked if this process strictly paralleled the inhibition of EGF degradation by chloroquine. Alternatively, the nuclear accumulation of EGF could have been due to some other effect of the inhibitor, probably on the outer nuclear membrane. It was found that exposure to chloroquine ($75 \text{ } \mu\text{M}$) had no effect on, or increased by at most twofold, the amount of surface-bound ^{125}I -EGF (measured by its release by trypsin). However, chloroquine-treated cultures contained as much as 19.3 times more immunoprecipitable and hence, presumably intact ^{125}I -EGF in their cell lysates than control cells (Table 1). This increase was directly paralleled by a 19-fold increase in the amount of nuclear-associated ^{125}I -EGF. As in the lysate of chloroquine-treated cells, 75% of the nuclear-bound ^{125}I -EGF could be precipitated with anti-EGF after solubilization from the nuclei. The results of these experiments suggest that in control cells, due to rapid degradation of the internalized EGF, only a small percentage (1%) of the internalized growth factor could be detected in the nucleus. In contrast, in chloroquine-treated cultures EGF degradation is greatly diminished, thus allowing a cytoplasmic accumulation of

EGF and making a greater proportion of the internalized, intact EGF available for nuclear binding.

The nature of the nuclear sites which interact with ^{125}I -EGF was examined next. Scatchard analysis of the ^{125}I -EGF nuclear accumulation (Fig. 2) shows that the observed nuclear binding is saturable and of high affinity. Two distinct classes of binding affinity are apparent: a high affinity component with a K_d of 0.28 nM and a lower affinity class of sites possessing a K_d of 2.7 nM . The binding capacity of each class of sites estimated from the Scatchard plots is 10^3 and 1.2×10^4 sites per nucleus, respectively. Interestingly, this observation is similar to findings regarding the nuclear binding of NGF in PC12 cells. In that system, two classes of binding sites which differ in affinity by an order of magnitude were also observed⁸, although the number of high-affinity sites was two- to fivefold greater than that seen for EGF in GH₃ cells. The NGF nuclear receptor sites in PC12 cells are resistant to extraction with Triton detergents, suggesting that they may be localized at the inner membrane⁸. In that system, both intact nuclei and isolated nuclear membranes retain the ability to bind ^{125}I -NGF. In the case of GH₃ cells, nuclei isolated from either untreated or chloroquine-treated GH₃ cells showed no detectable binding of ^{125}I -EGF when the growth factor was added *in vitro*. Similar negative results were

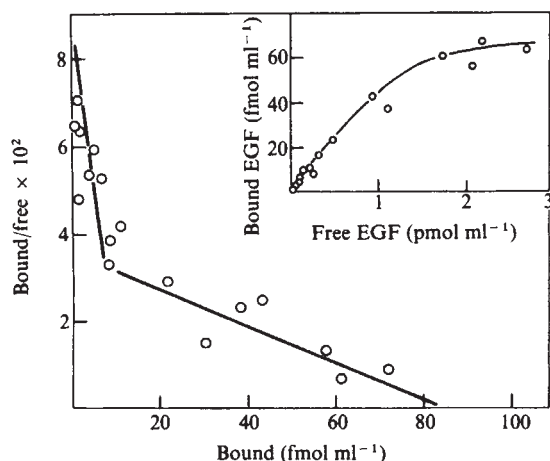


Fig. 2 Scatchard analysis of ^{125}I -EGF binding to GH₃ cell nuclei. Cells were seeded and media replaced as described in Fig. 1. Cultures were then incubated for 18 h at 37 °C in the presence of 75 μM chloroquine and increasing concentrations of ^{125}I -EGF (0.05–50 ng ml $^{-1}$) with or without 2.5 μg ml $^{-1}$ unlabelled EGF. Nuclei were analysed for the amount of specifically bound EGF as described in Fig. 1 legend. The extracellular concentration of free, intact EGF was determined by double immunoprecipitation (rabbit anti-EGF antiserum followed by goat anti-rabbit IgG) of an aliquot (100 μl) of the culture medium before the cultures were washed, as described in Table 1. Plates without cells were used to control for spontaneous breakdown of ^{125}I -EGF during the incubation period at 37 °C. Up to 20% of the total radioactivity was no longer immunoprecipitable, after incubation without cells, as opposed to only 2% in freshly labelled EGF.

obtained with detergent-washed nuclei (nuclei lacking the outer membrane) or with nuclei prepared by mechanical disruption and centrifugation through 2.0 M sucrose⁸ (nuclei with an intact outer membrane). We therefore examined the sub-nuclear localization of EGF by subjecting isolated nuclei with bound ^{125}I -EGF to enzymatic fractionation of the chromatin.

GH₃ cells were labelled for 24 h with ^{14}C -thymidine followed by a 12-h incubation with ^{125}I -EGF in the presence of chloroquine. The nuclei were then isolated and incubated with micrococcal nuclease at 22 °C. At various times, aliquots were taken and the amount of ^{125}I and ^{14}C released into the supernatant

determined. As shown in Fig. 3, micrococcal nuclease solubilized approximately 90% of the labelled DNA within 10 min. The solubilized DNA fragments showed the characteristic nucleosome repeat pattern of chromatin and contained DNA fragment sizes from monomers (180 base pairs) to octamers (Fig. 3b). However, no ^{125}I -EGF was released by the nuclease above that which dissociated during the course of the experiment (approximately 9% in the zero time controls), nor was any spontaneous release observed when the EGF-loaded nuclei were lysed with 1.0 mM EDTA. This suggests that the bound EGF is not localized in the chromatin fraction of the nucleus but rather is associated with the inner nuclear membrane or nuclear matrix fraction. The latter contains the so-called 'nuclear scaffolding' (ref. 15), structures thought to participate in the higher-order condensation and packaging of chromatin into metaphase chromosomes before cytokinesis¹⁶. Direct EGF binding to receptors in the inner nuclear membrane seems unlikely, however, as nuclei isolated by detergent showed no binding when analysed *in vitro* despite their retention of the inner membrane. These findings are in contrast to the sub-nuclear location of two other regulators of growth hormone and prolactin mRNA synthesis, namely the thyroid and glucocorticoid hormone receptors⁵. Both of these receptors are liberated from GH cell chromatin upon DNase digestion¹⁷ and in the case of T₃ there may be a preferential localization of the receptor in nuclear fractions more active in RNA synthesis^{18,19}.

Although a number of common steps appear to follow the initial cell surface binding of various polypeptide hormones and mitogens, the mechanism by which the respective cellular responses are exerted is still far from being understood. Some of these responses require a prolonged exposure to the effector, while others are temporary and occur within minutes. If internalization is a prerequisite, at least for the induction of long-term responses, then an interaction of either the intact polypeptide or its degradation product with the cell nucleus should be considered. Nuclear binding sites for insulin²⁰ and NGF⁸ have been identified in rat liver cells and PC12 cells, respectively. In the case of GH₃ cells, although binding to isolated nuclei has not yet been observed, a translocation pathway leading to a nuclear accumulation of EGF can be described in accordance with the present observations.

The nuclear accumulation of EGF in GH₃ cells was a small percentage of the total internalized EGF (1%) but increased markedly when the lysosomal degradation of the growth factor

Fig. 3 Influence of micrococcal nuclease on release of ^{125}I -EGF and ^{14}C -thymidine - labelled DNA from isolated GH₃ nuclei. Cells (1.5×10^7) were labelled for 24 h with 25 μCi (5 μCi ml $^{-1}$) ^{14}C -thymidine, followed by an additional 18-h incubation with ^{125}I -EGF (12.5 ng ml $^{-1}$) in the presence of 75 μM chloroquine. After isolation of the nuclei, aliquots were incubated at 22 °C with 40 units ml $^{-1}$ micrococcal nuclease (Worthington) for the indicated times. The reaction was stopped and nuclei lysed by the addition of a 10-fold volume of 1 mM EDTA and unreleased material was removed by centrifugation (15,000g, 5 min). ^{125}I -EGF in the two fractions (pellet and supernatant) was counted first. Each was subsequently brought to 1.0 M perchloric acid, heated to 70 °C for 30 min, and cooled on ice. Precipitated protein was removed by centrifugation and ^{14}C detected by scintillation counting in a Triton-toluene-omnifluor cocktail. The per cent of each label released was determined by dividing the amount of label present in the supernatant by the total (supernatant plus pellet) for each time point. The distribution of DNA fragments in the supernatant was examined by subjecting equal ^{14}C c.p.m. to fractionation on a 1% agarose gel (b). Outside lanes are marker DNA restriction fragments from plasmid pBR322 while the inside lanes are from the 0.5, 1, 1.5, 2, 3, 4 and 12 min time point supernatants (right to left). ○, Thymidine; □, EGF.

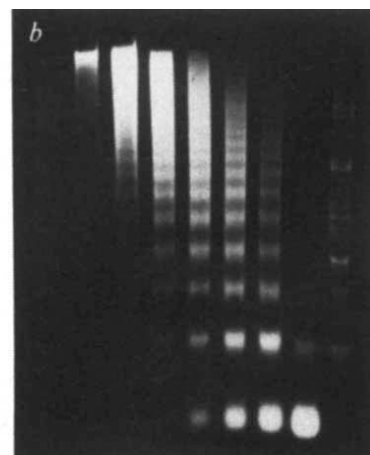
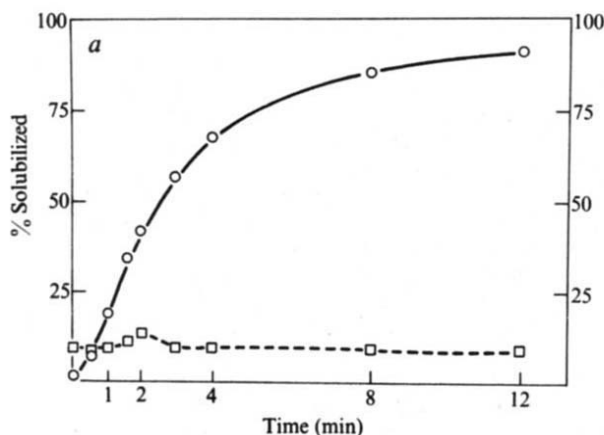


Table 1 Effect of chloroquine on degradation and subcellular distribution of EGF in GH₃ cells

Condition*	EGF concentration (fmol per 10 ⁶ cells)		
	Surface†	Lysate‡	Nuclei§
Control	5.07	1.88	0.303
Chloroquine (75 µM)	5.72	36.40	5.78
Increase (–fold)	1.12	19.3	19.0

* 10⁶ cells were incubated (18 h, 37 °C) with 2 ng ml⁻¹ ¹²⁵I-EGF, with or without chloroquine, in the presence and absence of 2.5 µg ml⁻¹ unlabelled EGF.

† Trypsin-releasable radioactivity. Washed cultures were treated with STV (0.05% trypsin and 0.02% EDTA Gibco) for 5 min at 22 °C, further proteolysis inhibited by adding calf serum (10%), and the radioactivity measured in an aliquot of the supernatant after centrifugation (330g, 5 min).

‡ Amount of immunoprecipitable ¹²⁵I-EGF present in the NP40 lysate minus cell-surface-associated (trypsin-releasable) ¹²⁵I-EGF. Immunoprecipitation was carried out by incubating (2 h, 37 °C) 1 ml of cell lysate with rabbit anti-EGF antiserum (3 µl) followed by addition (1 h at 37 °C and 16 h at 4 °C) of 10 µl goat anti-rabbit IgG antiserum (35% ammonium sulphate cut). Precipitable radioactivity (intact EGF) amounted, after correction for spontaneous breakdown of ¹²⁵I-EGF (Fig. 2 legend), to about 95% and 50% of the total radioactivity present in the lysates of chloroquine-treated and untreated cells, respectively. When tested in the tissue culture medium the amount of non-precipitable radioactivity did not exceed (in chloroquine-treated cultures) that found in the absence of cells, whereas in untreated cells release of ¹²⁵I-EGF degradation products, above the 'no cells' control, accounted for up to 90% of the amount of intact EGF accumulated in chloroquine-treated cells.

§ Nuclear pellets were dissolved in 9 M urea, insoluble material removed by centrifugation, and the supernatant (20 µl) subjected to immunoprecipitation as described above after dilution to 1.0 ml with phosphate-buffered saline. The percentage of immunoprecipitable radioactivity in nuclei prepared from chloroquine-treated and untreated cells was similar (65–75%).

was inhibited. This could occur through internalization of EGF by adsorptive endocytosis, followed by fusion of accumulated endocytotic vesicles with the outer nuclear membrane. Release of EGF, either bound or unbound to its surface receptor, into the perinuclear space, inner nuclear membrane or nuclear scaffolding structures could then follow. Such a process may be reflected by findings showing that cytoplasmic vesicles containing fluorescein-conjugated EGF form a perinuclear ring in A-431 cells²¹. In the immature rat uterus, fusion of lysosomal vesicles with the nuclear membrane has been considered as a possible means of introducing oestrogens into the nucleus²².

No regulatory action of a protein hormone has yet been shown to require hormone degradation. On the contrary, results from our laboratory demonstrate a full induction of thymidine incorporation and cell proliferation by EGF, despite the inhibition of its degradation by either leupeptin or antipain²³. Whether or not the nuclear accumulation of either intact or modified EGF is in fact responsible for the various nuclear effects exerted by EGF on GH₃ cells requires further investigation.

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VIP occurs in intrathyroidal nerves and stimulates thyroid hormone secretion

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Vasoactive intestinal polypeptide (VIP) is known to have powerful effects on the secretion from several endocrine and exocrine glands^{1–3}, and occurs in nerves with a ubiquitous distribution in the body^{4,5}. This infers that neuronal VIP may be a regulator of such secretion, and there is evidence that it is involved in the regulation of exocrine pancreatic function³. Previous studies have shown that adrenergic and cholinergic nerves participate in the regulation of thyroid hormone secretion^{6–9}. We describe here combined immunohistochemical and immunochemical studies which show that the thyroid of several species is supplied with VIP-containing nerve fibres that surround blood vessels and run between and along thyroid follicles and that in the mouse neuronal VIP participates in the regulation of thyroid hormone secretion through a mechanism that is mediated by cyclic AMP.

Thyroid tissue specimens from cat, pig and man were fixed by immersion in ice-cold 4% formalin buffered to pH 7.0. The human material was obtained at surgery for benign autonomous nodules. Mice, rats and guinea pigs were anaesthetized with sodium pentobarbitone or diethyl ether and perfused via the ascending aorta with large volumes of the fixative solution. The thyroid glands were dissected out and stored in the fixative for 24–48 h and rinsed for 24 h in an ice-cold Tyrode solution containing 5% sucrose. The tissue specimens were frozen in dry ice and sectioned in a cryostat at –15 °C. Sections, cut at 8 µm, were processed for the immunocytochemical demonstration of VIP, using an antiserum (5603) raised against highly purified porcine VIP¹⁰. The dilution of the antiserum was 1:80. The site of antigen-antibody reaction was revealed by the indirect immunofluorescence method of Coons *et al.*¹¹. Fluoresceinated goat anti-rabbit IgG was from SBL, Stockholm, Sweden. Controls were sections exposed to VIP antiserum which had been inactivated by incubation with pure porcine VIP (0.3–30 nmol per ml diluted antiserum) for at least 24 h.

The concentration of VIP in the thyroid was determined by radioimmunoassay. Thyroid glands were dissected out from anaesthetized cats, mice, pigs and rats. Normal thyroid gland tissue was also obtained from patients undergoing surgery for thyroid adenoma. The number of specimens are given in Table 1. The tissue was immediately frozen on dry ice, weighed and stored at –20 °C until extraction. VIP was extracted by homogenization at 0 °C in a glass homogenizer containing 5 vol of

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Table 1 Concentration of VIP (pmol per g wet weight) in the thyroid gland of various mammalian species

	Mouse	Rat	Cat	Pig	Man
Mean	25.1	28.6	16.6	0.8	1.8
s.e.m.	2.1	6.2	2.6	0.1	0.4
n	12	6	6	5	6

n, No. of glands analysed in each species.

acidified ethanol (70% ethanol containing 0.74% HCl, w/v). After centrifugation (1,500g) at 4 °C for 15 min, the supernatant was decanted and the residues resuspended and homogenized with additional 5 vol of acidified ethanol. After centrifugation, the combined supernatant fractions were evaporated to dryness *in vacuo*. The samples were reconstituted and diluted in assay buffer (sodium phosphate buffer, 0.4 mol l⁻¹, pH 7.4, containing 58 µmol human serum albumin (Behringwerke) and sodium chloride (0.1 mol l⁻¹) and assayed in at least three different dilutions. The concentration of VIP in the extracts was measured radioimmunochemically using antiserum no 5603-6 (refs 10, 12).

For studies on the effect of VIP, female mice of the NMRI strain (Anticimex) weighing 20–25 g at the time of experiments, were used. They were fed a standard pellet diet or a low-iodine diet (Astra-Ewos), and tap water *ad libitum*. Porcine VIP, bovine thyrotropic hormone (TSH; Ferring AB) or saline was injected intravenously (i.v.) and the animals were killed after 30 min. The thyroid glands were dissected out, fixed for 18 h at 4 °C in Bouin's solution, dehydrated with ethanol, embedded in

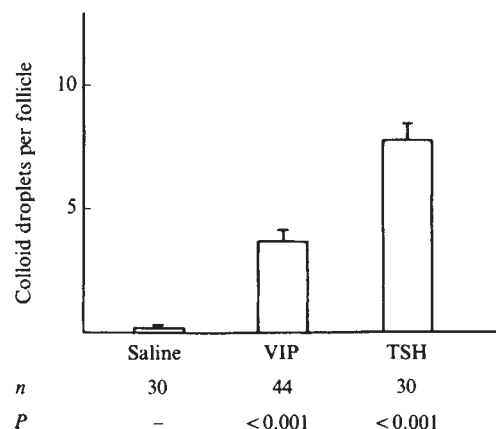


Fig. 2 Colloid droplets in thyroid follicle cells 30 min after an i.v. injection of saline, VIP or TSH. Values are mean ± s.e.m. P, Probability level of random difference. n, No. of follicles counted.

paraffin, sectioned at 6 µm and stained by the periodic acid-Schiff (PAS) technique. This technique stains colloid in the follicle lumen as well as colloid droplets in the follicle cells⁷. Radioiodine release from the thyroid was assessed according to McKenzie¹³, as modified by Rerup and Melander¹⁴. The animals were given a low-iodine diet 10 days before experimentation to ensure adequate thyroidal uptake of radioiodine. Each animal was injected intraperitoneally (i.p.) with 16 µCi Na¹²⁵I (Radiochemical Centre) three days before the experiments, and was given 20 µg L-thyroxine (British Drug Houses Ltd) subcutaneously (s.c.) 72 and 24 h before the experiments. Blood (100 µl) was sampled by orbital puncture before and at specified intervals after the i.v. injection of VIP, TSH and saline, respectively. The injection volumes were 0.1 ml (s.c. and i.p.) or 0.2 ml (i.v.). The radioactivity was measured in an autogamma spectrometer.

The influence of VIP and TSH on the production of cyclic AMP in the thyroid was investigated according to Åhrén *et al.*¹⁵, with an ion exchange step included. Briefly, the animals were killed by neck dislocation. The thyroid lobes were dissected out and placed in tubes with 1 ml Krebs-Ringer bicarbonate buffer containing 10 mmol l⁻¹ glucose. The preincubation lasted for 30 min and the incubation 25 min. During preincubation and incubation, the medium was bubbled with 95% O₂/5% CO₂ to ensure constant pH and oxygenation. To reduce degradation of newly formed cyclic AMP, theophylline (1.0 mmol l⁻¹) was added. Cyclic AMP content was determined in the tissue and in the incubation medium. The tissue was homogenized in ice-cold 10% trichloroacetic acid and centrifuged at 1,500g at 4 °C for 30 min. The supernatant was passed through a Dowex-50 column with 1.5 ml bed volume and eluted with distilled water. The eluate was freeze-dried, redissolved in 500 µl distilled water and analysed for its cyclic AMP content by the method of Gilman¹⁶, as modified by Tovey *et al.*¹⁷. The cyclic AMP content was expressed as pmol per mg tissue protein, determined by the method of Lowry *et al.*¹⁸. The production of cyclic AMP was calculated as the sum of tissue and medium content. ³H-cyclic AMP was from NEN and cyclic AMP-dependent protein kinase from Sigma. All commercial chemicals were reagent grade.

Immunoreactive nerve fibres were observed in the thyroid of all species examined. They were most frequent in mouse, less frequent in rat and cat and scarce in guinea pig, pig and man. The nerve fibres were thin and beaded, occurred both around blood vessels (Fig. 1a) and between and along thyroid follicles (Fig. 1b). In the cat and mouse the fibres were rather uniformly distributed within the gland, whereas in the rat they had a characteristically patchy occurrence with areas totally devoid of immunoreactive fibres. In man, single fibres were observed in the stromal tissue surrounding follicles.

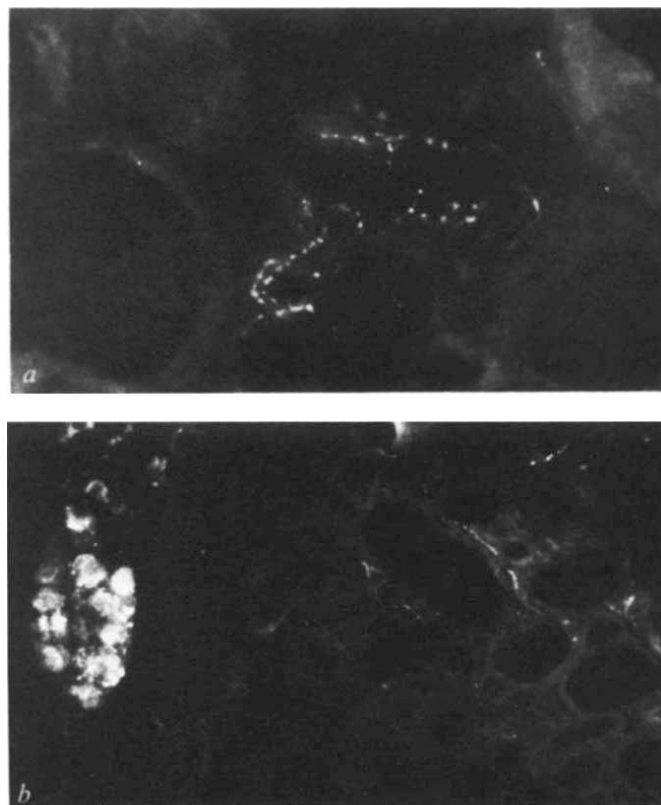


Fig. 1 Immunohistochemical demonstration of VIP nerves in rat (a) and mouse (b) thyroid. VIP nerves were distributed both around blood vessels (a) and between and along thyroid follicles (b). In the mouse, VIP immunoreactivity was seen in cell bodies in a local ganglion (left of b). Magnification a, ×125; b, ×190.

In the mouse, a small ganglion containing VIP-immunoreactive nerve cell bodies and fibres was regularly encountered close to the thyroid capsule at the dorso-medial surface of each lobe (Fig. 1b). The ganglion was sometimes seen to issue a bundle of immunoreactive fibres projecting into the thyroid parenchyma. This suggests a local origin of at least some of the VIP fibres.

The concentrations of VIP in the thyroid gland of five mammalian species are given in Table 1. Concentrations measured by radioimmunoassay roughly agreed with the relative number of VIP-containing nerve fibres found by immunohistochemistry.

Injection of VIP, like TSH, was associated with formation of numerous intracellular colloid droplets in the mouse thyroid. The increase in the number of colloid droplets was significant compared to saline-injected controls (Fig. 2). VIP, like TSH, raised the blood ^{125}I levels in mice pretreated with ^{125}I and thyroxine. For both VIP and TSH, maximum radioiodine concentrations were reached at 2 h (Fig. 3). Therefore, in subsequent experiments, samples were collected at 0 and 2 h. Figure 3 shows the responses to different doses of VIP or TSH, and to their combination. The dose-response curve for VIP was less steep than that for TSH. The response to 5.0 nmol VIP was

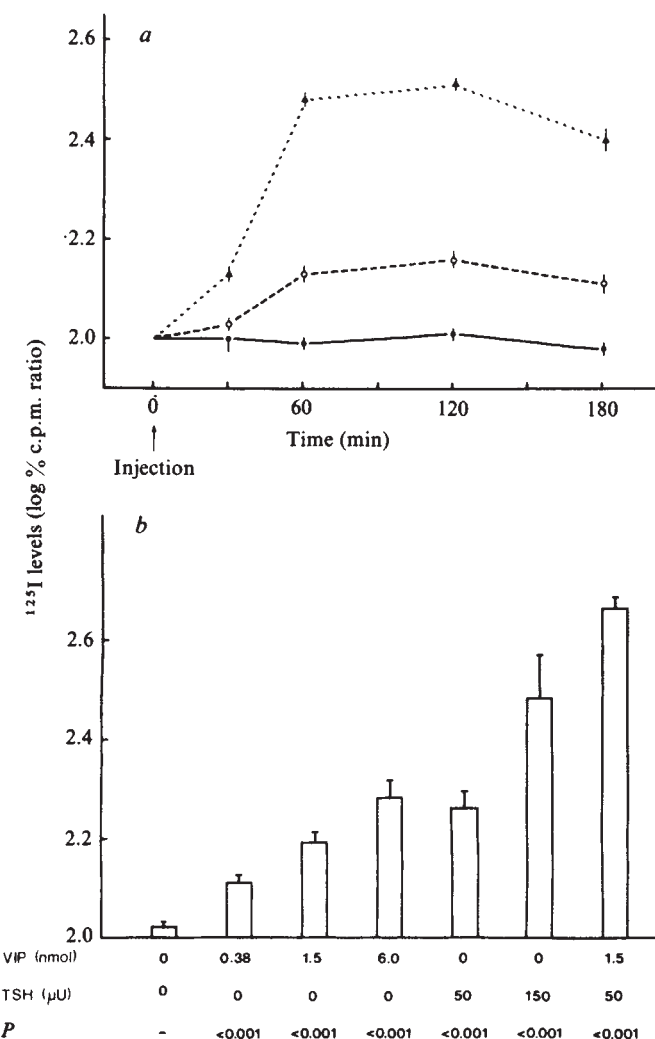


Fig. 3 Changes in blood radioactivity in mice pretreated with ^{125}I and thyroxine following injection of VIP, TSH and saline, respectively. *a*, Time course of the changes. 5–7 mice per group. ▲, TSH (50 µU); ○, VIP (1.5 nmol); ●, saline. *b*, Dose-response relationship. 8–17 mice per group. log % c.p.m. ratio of radioactivity before and 2 h after injection. Values are mean $\pm$ s.e.m. *P*, Probability level of random difference.

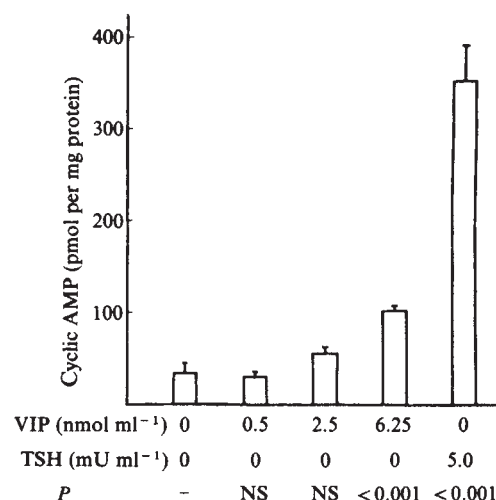


Fig. 4 Thyroidal production of cyclic AMP (pmol per mg tissue protein) *in vitro* during 25 min exposure to VIP or TSH. Four observations per group. *P*, Probability level of random difference. NS, Not significant.

roughly equivalent to that to 65 µU TSH. VIP (5.0 nmol) and TSH (50 µU) potentiated the effect of each other. Most, if not all, thyroidal effects of TSH are thought to be mediated^{19,20} by cyclic AMP. Like TSH, VIP increased the cyclic AMP production, although to a lesser degree (Fig. 4). VIP has previously been shown to stimulate cyclic AMP formation in several tissues^{21–23}.

The findings that VIP occurs in nerve fibres in the thyroid and has a stimulatory effect on thyroid hormone secretion suggest that neuronal VIP participates in the regulation of thyroid activity. Earlier studies have revealed that both adrenergic and cholinergic nerves are involved in the regulation of thyroid activity^{6–9}. The interaction between the various nervous mechanisms in the control of thyroid function remains to be elucidated.

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Intracellular studies showing modulation of facial motoneurone excitability by serotonin

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The application of serotonin to certain myenteric plexus neurones in the guinea pig small intestine causes a slow depolarization of membrane potential, accompanied by increased neuronal excitability and input resistance¹. On the other hand, microiontophoretic application of large amounts of serotonin onto mammalian spinal motoneurons is reported to cause membrane hyperpolarization and decreased excitability². However, on the basis of recording spinal reflex activity, serotonin has been reported to enhance net motoneurone activity³. Moreover, studies using extracellular single-cell recording techniques indicate that serotonin in small amounts facilitates synaptically or glutamate-induced excitation of mammalian motoneurons in the facial nucleus⁴ and spinal cord⁵. It was suggested that these facilitatory actions were modulatory in nature, as serotonin did not induce motoneurone spiking in the absence of extrinsic excitatory input. The study reported here investigated the membrane mechanisms underlying these modulatory effects by obtaining intracellular recordings from rat facial motoneurons during extracellular microiontophoretic application of serotonin, methysergide (a serotonin antagonist) and noradrenaline. Serotonin caused a slow depolarization of membrane potential of about 5 mV which remained subthreshold, accompanied by an increase in electrical excitability of the neurone, and an increase in input resistance. Noradrenaline caused the same changes. Methysergide antagonized the effects of serotonin, but not noradrenaline, indicating that these actions of serotonin are selective and receptor mediated.

Figure 1 illustrates the changes which occurred in a rat facial motoneurone with the iontophoretic administration of a relatively low dose (+25 nA ejection current) of serotonin. Within 32 s, the resting potential shifted from -68 mV to -63.5 mV (Fig. 1b), input resistance increased by 32% from an average value of 4.7 M Ω to one of 6.2 M Ω (Fig. 1c), and a constant current intracellular depolarizing pulse (1.1 nA, 182 ms duration) which had been adjusted to 73% of rheobase became effective in triggering action potentials (Fig. 1a, d), indicating an increase in excitability of the neurone.

Increased excitability and slow depolarization in response to iontophoretically applied serotonin were seen in 35 of 41 facial motoneurons analysed. The maximal depolarization seen was 8 mV with 50 nA of serotonin applied for 2 min. The typical amount of depolarization was 3–6 mV with iontophoretic currents of 10–60 nA applied for 0.5–2.0 min. Changes in input resistance were more difficult to detect, due in part to fluctuations in electrode resistance which caused imbalances in the bridge circuit adjustment. However, all changes in input resistance which were clearly seen were increases. No decreases in input resistance were detected in response to iontophoretically applied serotonin. Excitability changes in response to very low doses of serotonin were particularly evident when the amplitude of the long (130–200 ms) depolarizing pulse was adjusted to rheobase so that only one action potential was triggered from it. In such cases, iontophoretic currents of serotonin as low as 5 nA

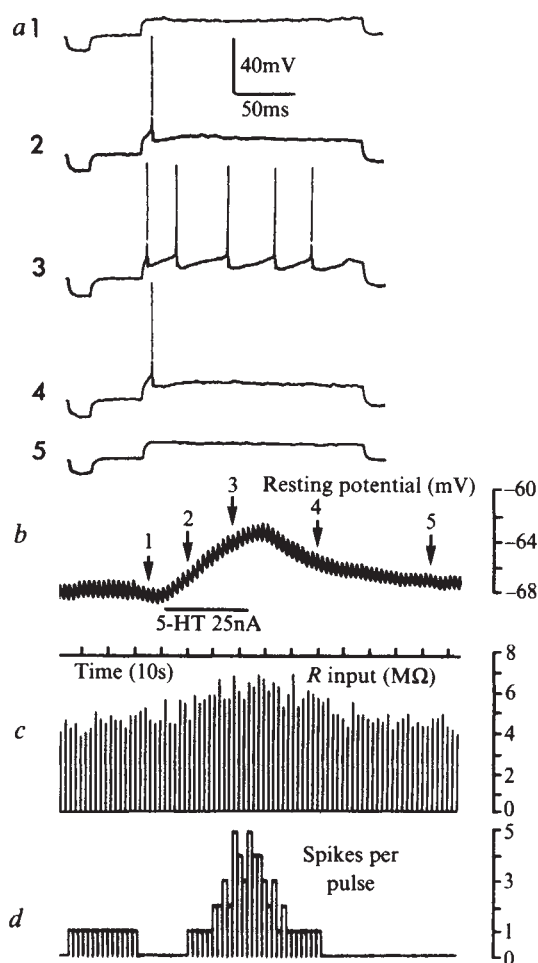


Fig. 1 Effects of iontophoretically applied serotonin (5-HT) on membrane resting potential, input resistance and excitability of a rat facial motoneurone. *a*–*e*, Selected sweeps of the oscilloscope taken before, during and after serotonin iontophoresis. Each sweep consisted of a 1.0-nA hyperpolarizing pulse used for input resistance measurement, followed by a longer depolarizing pulse. The depolarizing pulse was set at 1.1 nA, which was 73% of rheobase. Sweeps occurred once every 1.9 s. *b*–*d*, Simultaneous records of resting membrane potential (*b*), input resistance (*c*) and number of spikes triggered by the depolarizing pulse (*d*). Time marker occurred once every 10 s and applies to *b*–*d*. The numbered arrows in *b* indicate the points in time at which the numbered sweeps in *a* were taken. The bar in *b* indicates the time during which serotonin (25 nA) was iontophoretically applied. The saw-toothed appearance of the trace in *b* was due to deflections of the pen that remained after filtering, caused by the depolarizing pulse. Input resistance was determined by transferring the stored data from FM tape to a storage oscilloscope where each hyperpolarizing pulse was displayed at high gain and fast time base. The voltage displacement was carefully measured, with compensation for any imbalance in the bridge circuit. Calculated values for input resistance are shown graphically in *c*. The 14 individual spikes in *d* preceding the onset of the serotonin were caused by the depolarizing pulse set at rheobase, before being adjusted to 73% of rheobase. Intracellular recording micropipettes were filled with 2 M KCl and had resistances of 15–25 M Ω . These were affixed with dental acrylic to five-barrel iontophoretic micropipettes, with tip separations between recording and iontophoretic electrodes of 25–60 μ m. Iontophoretic barrels were filled with serotonin creatinine sulphate (Regis, 40 mM, pH 4.0), methysergide maleate (Sandoz, 5 mM, pH 4.0), (–)noradrenaline bitartrate (Regis, 100 mM, pH 4.0) and NaCl (2 or 4 M) which was used for the current balance channel such that net current flow from the iontophoretic electrodes was zero at all times. Retaining currents of -15 nA were applied to drug barrels between periods of ejection. The facial nucleus was located in anaesthetized (chloral hydrate, 400 mg per kg, intraperitoneal) male albino rats by the presence of a characteristic antidromic field potential¹⁴ in response to electrical stimulation of the zygomatic branch of the facial nerve.

applied for 30 s caused multiple spikes to be triggered from the depolarizing pulse (not shown).

Figure 2 illustrates the selective antagonism by methysergide of the serotonin induced increase in motoneurone excitability. A depolarizing pulse (5.4 nA intensity, 132 ms duration, Fig. 2a) was delivered intracellularly to a facial motoneurone once every 2.1 s. This pulse had been adjusted to 90% of rheobase. With the iontophoretic administration (40 nA for approximately 1 min) of either serotonin (Fig. 2b₁) or noradrenaline (Fig. 2c₁), excitability was increased, as indicated by the fact that the depolarizing pulses triggered multiple spikes. However, in the presence of iontophoretically applied methysergide (7 nA) and with the same current of serotonin, no spikes were triggered from the depolarizing pulse during 1 min (Fig. 2b₂), indicating a greatly reduced effect of serotonin on excitability. Methysergide had only a small effect on responses to noradrenaline (Fig. 2c₂), and this may have been due to a slight decrease in excitability caused by the methysergide itself.

The mechanism for this methysergide-induced decrease in motoneurone excitability was not explored, although we did find that iontophoretically applied methysergide, as well as intravenously administered methysergide or cyproheptadine (another serotonin antagonist), cause decreases in motoneurone excitability, determined by rheobase measurements. It is possible that the serotonin blockers antagonize a tonic facilitatory serotonin input, as facial motoneurons are known to receive a dense serotonin innervation^{4,6,7}. A similar explanation has previously been proposed to explain the effects of the serotonin antagonist metergoline on spinal motoneurons recorded extracellularly⁵.

Our experiments, using intracellular recording techniques, agree with previous studies using extracellular single unit recording. These showed that microiontophoretically applied serotonin facilitates the excitation of motoneurons caused by stimulation of excitatory afferents or by the microiontophoretic application of glutamate, but that serotonin alone does not elicit action potentials^{4,5}. We have shown that this modulatory action of serotonin is associated with a slow, subthreshold depolarization which is accompanied by an increase in both electrical excitability and input resistance. The ionic mechanisms for these serotonin-induced membrane changes are not known, but similar changes in guinea pig small intestine neurones are postulated to be caused by a decrease in Ca^{2+} -dependent potassium conductance (g_{K^+})⁸. Similarly, serotonin causes a depolarization of certain molluscan neurones by decreasing g_{K^+} (ref. 9).

Because we measured excitability directly by means of intracellular injection of depolarizing current, we conclude that serotonin (and noradrenaline) causes a generalized increase in excitability to all types of excitatory input, rather than causing specific increases in receptor sensitivity to a particular neurotransmitter (for example, glutamate). Evidence for selective effects of noradrenaline on γ -aminobutyric acid but not glycine 'postsynaptic mechanisms' in cerebellar Purkinje cells has been reported¹⁰. Although our data do not rule out possible additional actions of serotonin on various pre- and postsynaptic receptors, the slow depolarization and increased excitability caused by serotonin in facial motoneurons cannot be due to increased tonic release of or increased sensitivity to a conventional excitatory neurotransmitter which would act by increasing ionic conductance to depolarize the membrane. If this were the case, input resistance would decrease rather than increase. Rather, serotonin seems to act via its own receptor to change certain membrane conductances (perhaps g_{K^+}), resulting in a generalized increase in motoneurone excitability. Such a general physiological action is consistent with results from electron microscopic autoradiographic studies which show that ³H-serotonin-labelled terminals form direct axosomatic and axodendritic synaptic junctions on facial motoneurons¹¹. A generalized facilitation of excitatory inputs to motoneurons would account for some of the diverse motor components of the excitatory behavioural '5-HT syndrome' seen in animals

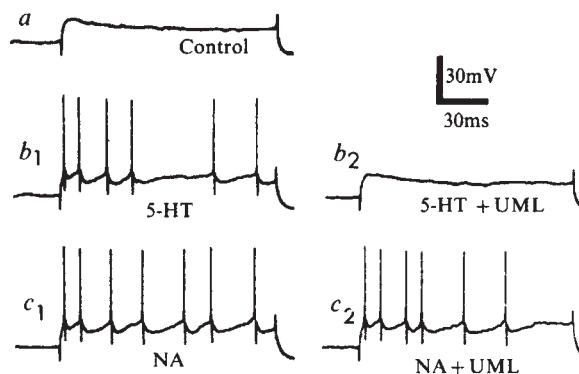


Fig. 2 Selective antagonism of the serotonin (5-HT)-induced increase in excitability by methysergide (UML). *a*, A depolarizing pulse (5.4 nA, 132 ms), which was adjusted to 90% of rheobase, did not elicit any action potentials. *b*₁, In the presence of iontophoretically applied serotonin (40 nA for 56.5 s) the depolarizing pulse elicited multiple spikes, indicating increased excitability of the neurone. *c*₁, Similarly in the presence of noradrenaline (NA, 40 nA for 55.3 s) multiple spikes were triggered by the depolarizing pulse. Three minutes were allowed for recovery, between the offset of serotonin and onset of noradrenaline. *b*₂, After methysergide had been on continuously for 3.5 min (7 nA), iontophoretically applied serotonin (40 nA for 59.5 s) did not cause spikes to be triggered by the depolarizing pulse. *c*₂, Iontophoretically applied noradrenaline (40 nA for 56.0 s) still caused multiple spikes to be triggered by the depolarizing pulse, even though the methysergide had been on continuously for 8.5 min. Four minutes were allowed for recovery from the effects of the serotonin before turning on the noradrenaline. To control for changes in methysergide-induced excitability, we ran a second series of tests (data not shown) on the same neurone, in which the depolarizing pulse was adjusted to compensate for changes in rheobase. After 10 min of iontophoretically applied methysergide (7.3 nA), rheobase had increased 13%, indicating decreased excitability. The amplitude of the depolarizing pulse was then adjusted to 90% of the new rheobase, and the effects of serotonin and noradrenaline determined as before. The results were essentially the same as those shown above, except that this time there was not even a small reduction in the effectiveness of the noradrenaline. In fact, the number of spikes triggered was actually slightly more than with noradrenaline alone, probably because of the larger amplitude of the depolarizing pulse. However, the serotonin-induced increase in excitability was strongly antagonized by methysergide, and no spikes were triggered by the depolarizing pulse in the presence of serotonin plus methysergide. Similar findings were obtained in two other neurones in which the depolarizing pulse amplitude was readjusted to compensate for methysergide-induced changes in rheobase.

injected with pharmacological agents which increase stimulation of postsynaptic serotonin receptors^{12,13}.

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Absence of potassium conductance in central myelinated axons

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Two voltage-dependent changes in ionic permeability are responsible for the action potential in squid giant axon¹. The depolarization phase of the action potential is due to an initial increase in sodium ion permeability, and repolarization is primarily the result of a later increase in potassium permeability. However, voltage-clamp experiments on mammalian peripheral nodes of Ranvier indicate that potassium conductances (g_K) may be minimal or lacking for intact mammalian peripheral myelinated axons²⁻⁴. Repolarization for these fibres has been explained in terms of a rapid sodium inactivation and large leakage current³. When the myelin around these fibres is acutely disrupted, an immediate and prominent g_K appears⁴. Following demyelination, g_K blocking agents have been shown to reduce late outward currents that are not present in normal myelinated fibres⁵. This suggests that K^+ channels are present in the axonal membrane under the myelin but are 'masked' in normal peripheral myelinated axons. Previous studies have not investigated the presence or role of K^+ channels in central myelinated axons. We here establish that g_K is not detectable in mammalian dorsal column axons.

The effects of superfusion of myelinated axons in the rat dorsal columns (DCs) with the voltage-dependent g_K blocking agents tetraethylammonium hydrochloride (TEA) and 4-aminopyridine (4-AP) were studied. We also recorded intra-axonally from the axons with glass microelectrodes filled with TEA. This allowed us to reach internodal as well as nodal axon membrane in the intact myelinated central axon. Although TEA injection into spinal motoneurons elicited a rapid and powerful prolongation of the motoneuron (soma-dendritic) action potential, the axonal action potential recorded from DC axons remained virtually unchanged even after prolonged TEA injection. In addition, superfusion of the spinal cord with 4-AP or TEA neither changed the action potential waveform nor altered the fibre refractory period.

Extra- and intra-axonal potentials were recorded from the DCs of the rat spinal cord following local DC or sciatic nerve stimulation. The animals were deeply anaesthetized with urethane (1.5 g kg⁻¹) and prepared for acute experimentation. An agar pool was built around the exposed lumbar spinal cord to allow for superfusion of the DC surface with oxygenated and warmed (38 °C) normal Ringer solution (NS) into which 4-AP or TEA could be added⁶. Recordings were confined to within 50 μ m of the surface of the spinal cord for the superfusion experiments.

Glass microelectrodes were filled with 3.0 M NaCl for field potential recordings and 2.0 M TEA for intracellular or intra-axonal recordings. Figure 1a shows the DC fibre field potential elicited from local surface stimulation of the DC superfused with normal Ringer. The early positive-negative component of the field corresponds to the collective action potential activity of fast conducting myelinated DC axons. The field remains virtually unchanged after introduction of 4-AP (Fig. 1b) or TEA (Fig. 1c) into the superfusion pool. When the potassium concentration of the NS was increased from 3.0–30 mM, a reversible conduction block occurred promptly, thus indicating the efficacy of the superfusion (Fig. 1d). In addition to not affecting the field

potential waveform elicited from a single stimulus, external 4-AP or TEA application also did not alter the refractory period of the fibres as determined from paired stimulation experiments (data not shown). In contrast to the absence of response of the myelinated DC fibres to superfusion with TEA and 4-AP, the compound action potential of nonmyelinated cerebellar parallel fibres is markedly altered by both of these agents⁷. Although 4-AP⁸⁻¹⁰, and in some instances TEA¹¹, have been shown to block voltage-dependent g_K by external application, the accessibility of either 4-AP or TEA to K^+ channel sites following external application is open to question. This is particularly important in view of the recent studies of Chiu and Ritchie⁴ showing that K^+ channel distribution may be inhomogeneous, with K^+ channels present at internodal or paranodal membrane regions but not at nodal membrane.

Prolongation of the somatic action potential has been reported¹²⁻¹⁴ following intracellular recordings from neuronal cell bodies with TEA electrodes. This effect appears rapidly after diffusion of TEA from the microelectrode or is facilitated by depolarizing currents. Figure 2a shows an action potential recorded from a motoneuron immediately after impalement with a TEA microelectrode. The responses shown in Fig. 2b and c were recorded 4.0 and 12.0 min later, respectively. The action potentials are much prolonged (compare Fig. 2b and c to a), indicating a block of g_K (ref. 12). The same microelectrode was used for recording the intra-axonal responses from DC axons shown in Fig. 2d-f. All electrode probes for DC axons were confined to the dorsal 200 μ m of the spinal cord, within 300 μ m of the spinal cord midline. Intra-axonal impalements were identified by: (1) the presence of negative resting potential; (2) the all-or-none character of the action potential with no underlying synaptic potential; and (3) when possible, collision of directly induced action potentials with those induced from

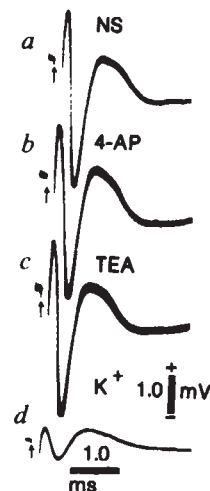


Fig. 1 Field potentials elicited from surface stimulation of the lumbar DC and recorded 3 mm anterior to the stimulation site within 20 μ m of the surface. *a*, Responses recorded when the spinal cord was continuously superfused with normal Ringer solution NS. The early positive-negative components of the field correspond to action potential activity of the fast conducting myelinated fibres of the DC. The responses in *b* and *c* were obtained after 30 min of superfusion in 4-AP (3.0 mM) and TEA (10 mM) solutions, respectively. Virtually no change occurs in the waveform of the early positive-negative field component, thus indicating the ineffectiveness of these voltage-dependent g_K blocking agents on these fibres. *d*, A response recorded within 2 min after the K^+ concentration was increased from 3 to 30 mM. A significant reduction in the field occurs at high K^+ concentrations. This effect is reversible within minutes after superfusion with NS. The K^+ blocking action indicates the efficacy of the superfusion system, and shows that the superfusate gains access to the nodal extracellular space. The calibration in *e* pertains to all records.

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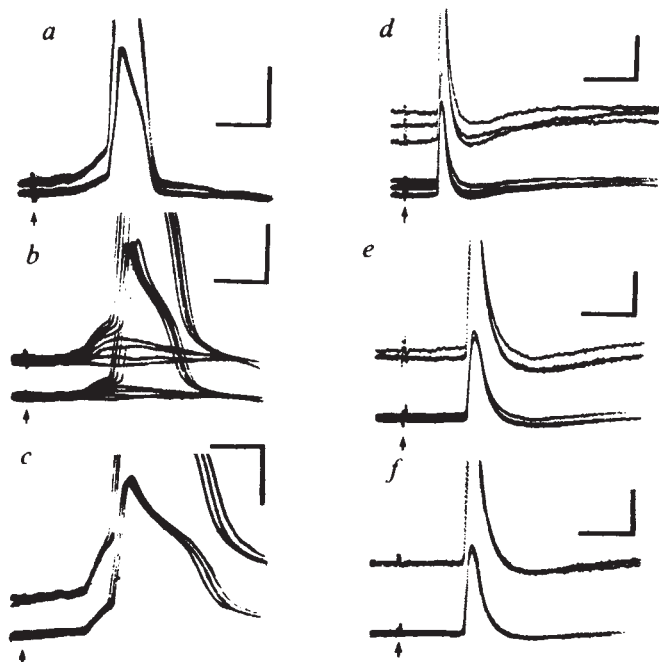


Fig. 2 *a-c*, Intracellular recordings obtained from spinal motoneurons with TEA microelectrodes following stimulation of the ipsilateral sciatic nerve. Orthodromic action potentials can be seen arising from an excitatory postsynaptic potential (EPSP). The upper traces correspond to high gain recordings and the lower traces to low gain recordings. *a*, Records obtained immediately after impalement of the motoneurone. *b*, Records obtained at about 4 min after impalement. Stimulation intensity was varied to demonstrate the graded nature of the EPSP and all-or-none properties of the action potential. Note the broadening of the action potential as the TEA diffuses into the cell. *c*, Records from same cell at about 12 min post-impalement. A marked prolongation of the action potential is evident. *d-f*, Intra-axonal action potentials recorded with same TEA microelectrode as used for *a-c*, from a DC axon near the midline surface of the lumbar cord. *d* and *e* show the axonal action potential at different oscilloscope sweeps, near the time of axonal impalement. *f*, Responses recorded about 25 min after impalement. Depolarizing currents were passed at the time between the records of *e* and *f*. No appreciable prolongation of the axon action potential can be seen after TEA injection. Vertical calibrations indicate 20 mV for lower traces and 10 mV for upper traces for *a-f*. Horizontal calibrations indicate 4 ms in *a-d* and 2 ms in *e* and *f*. Positive voltage deflection is upward.

sciatic nerve stimulation. Conduction velocities for these fibres were greater than 15 m s^{-1} , indicating their myelinated nature. After 25 min of impalement with a TEA microelectrode, virtually no change in the axonal action potential duration can be seen (Fig. 2*f*). We also passed depolarization pulses through the microelectrode to facilitate TEA entry, but still no change in action potential waveform occurred. For five axons, intra-axonal penetrations were held for over 45 min each. No significant change in spike waveform or refractory period was seen in these axons or in 26 other axons held for periods greater than 5 min but less than 45 min. During the passage of depolarization currents, the action potential amplitude was decreased, and hyperpolarization increased the action potential amplitude, thus indicating that the site of impalement was electrotonically close to the site of impulse origin.

The observation here, that external application of either TEA or 4-AP does not prolong the action potential of DC myelinated axons, extends observations made in peripheral nerves to DC axons in the mammalian central nervous system. Also, the lack of action potential prolongation or changes in refractory period after direct intra-axonal injections of TEA into these central myelinated axons suggests that K^+ channels contribute little to action potential waveform and recovery for these axons. One might question whether TEA effectively passed from our

microelectrode into the axons, and once inside if it diffused sufficiently to present itself to nearby internodal as well as nodal axon surfaces. However, other observations¹²⁻¹⁴ and this study show that TEA readily diffuses from electrode to cytoplasm of neurone somata. Although geometrical differences are present between soma-dendritic and axonal regions that could vary diffusion properties, the rapidity of the TEA action on large volume motoneurons, the facilitation of TEA ejection by depolarization pulses, and the relatively long duration impalements of the axons (several over 45 min), suggest that TEA may have passed over a significant axonal length.

Voltage-clamp experiments in mammalian peripheral nerve⁴ reveal an immediate appearance of g_{K} after acute myelin disruption, suggesting that K^+ channels are present under the myelin in normal myelinated axons. The absence of demonstrable g_{K} at intact mammalian peripheral nodes of Ranvier³ and the lack of effect on the action potential of direct intra-axonal injections of TEA into DC fibres suggest that g_{K} does not contribute significantly to spike electrogenesis in at least several types of intact mammalian myelinated fibres. Chiu and Ritchie (personal communication) have suggested that potassium channels in the internodal axon membrane may have a normal role in preventing repetitive firing at the node. Although we did not observe repetitive firing after TEA or 4-AP application, our experiments cannot rule this out.

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Spontaneous quantal events induced in toad rods by pigment bleaching

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Barlow proposed that absolute visual threshold is limited by photon-like noise events in the rod photoreceptors¹, and he later extended the idea to explain the elevation of threshold following bright bleaching lights in terms of increased noise in the photoreceptors². Rushton, on the other hand, has proposed that the threshold elevation during dark adaptation involves changes not within the photoreceptors themselves but rather in the gain of a subsequent 'pool'³. I report here measurements of outer segment current in individual rod photoreceptors which demonstrate that spontaneous fluctuations occur at a greatly increased rate following bleaches of around 1%, and that these fluctuations have the form expected for random occurrences of the single photon events. This is consistent with Barlow's ideas but does not indicate whether a gain change subsequent to the receptors also occurs.

The experiments were performed on toad rods using a suction pipette⁴⁻⁶. Briefly, a fully dark-adapted animal was pithed and one eye was enucleated under dim red light; then, using IR illumination, the retina was removed and cut into small pieces. With the aid of an IR-sensitive television camera system attached to an inverted microscope the pieces were manipulated under high magnification, and single rod outer segments were drawn into a fine glass pipette by gentle suction, so that membrane current could be measured and the outer segment stimulated with visible light. When recording spontaneous dark events the IR-light was turned off, and stringent precautions were taken to exclude stray light. As in the experiments of Lamb *et al.*⁸, two different Ringer solutions were used, one buffered with HEPES^{4,5,9-11} and the other with bicarbonate/CO₂ (ref. 12), both at pH 7.8. The observed noise increase on bleaching was similar in the two cases, but in HEPES-Ringer the cells gave slower and more sensitive responses which facilitated detection of quantal responses, while the bicarbonate/CO₂-Ringer was probably more physiological but gave faster, less sensitive responses⁸.

Figure 1 shows the effect in a cell recorded in HEPES-Ringer, with two traces before the bleach and one trace during recovery. In this Ringer the single photon event is quite large and, as reported by Baylor *et al.*⁷, spontaneous occurrences of the quantal events are apparent in the dark-adapted state. Within the 400 s of the first two records, ten such events (marked by arrows) can be seen, in good agreement with the reported mean of 1 per 50 s (ref. 7). The events have an amplitude of almost 1 pA and a time course similar to that of the average flash response to dim light. In the bottom trace, 3 min after a bleach of about 0.4%, the cell showed very marked current fluctuations, even though the flash sensitivity measured at this time had declined slightly from its pre-bleach level. The power spectrum of these fluctuations is shown in Fig. 2, and is compared with the power spectrum predicted for randomly occurring photon-like events. The inset shows the average flash response measured after the bleach, and has been fitted by the independent activation equation^{5,6,13} with $n=4$ stages, and $t_{\text{peak}}=1.75$ s. The power spectrum associated with this kinetic form consists of the product of $n=4$ lorentzians:

$$S(f) = S(0) / \prod_{k=1}^n \left[1 + \left(\frac{2\pi f \tau}{k} \right)^2 \right], \quad (1)$$

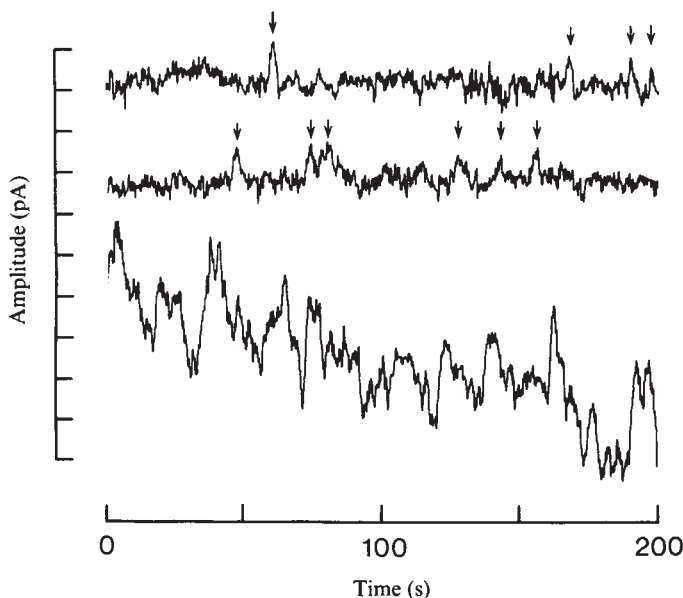


Fig. 1 Increased fluctuations following a bleach. Traces are 200 s records before (upper two traces) and 3 min after a bleach (lowest trace); arrows mark presumed quantal events. Bleach of about 0.4% (3.6×10^5 photons μm^{-2} , calculated to isomerize 9×10^6 rhodopsins); saturating response 18 pA; 23.0 °C; HEPES-Ringer. Each ordinate division represents 1 pA.

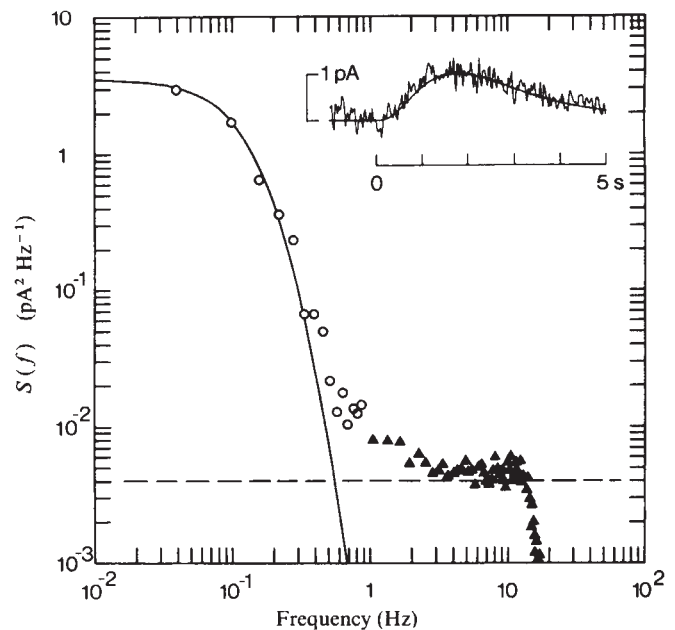


Fig. 2 Power spectrum of post-bleach fluctuations from cell of Fig. 1. Open symbols, averages of 3 raw frequency points; filled symbols, averages of 15. From five records, each 2,048 points at 25-ms intervals (51.2 s); filtered by 15 Hz 6-pole low pass filter and 10 s RC high pass filter. Continuous curve is equation (1) with $S(0)=3.5 \text{ pA}^2 \text{ Hz}^{-1}$; broken line is thermal noise of $4 \times 10^{-3} \text{ pA}^2 \text{ Hz}^{-1}$ for leakage resistance 4 M Ω . Inset shows average flash response determined from six flashes immediately before and six flashes immediately after the lowest record in Fig. 1.

with $\tau = t_{\text{peak}}/(n-1)$ and is shown by the continuous curve. The vertical position has been chosen to give the best fit by eye, and the broken line is the expected thermal noise. Figure 2 shows that the post-bleach fluctuations have the form expected for randomly occurring single photon events.

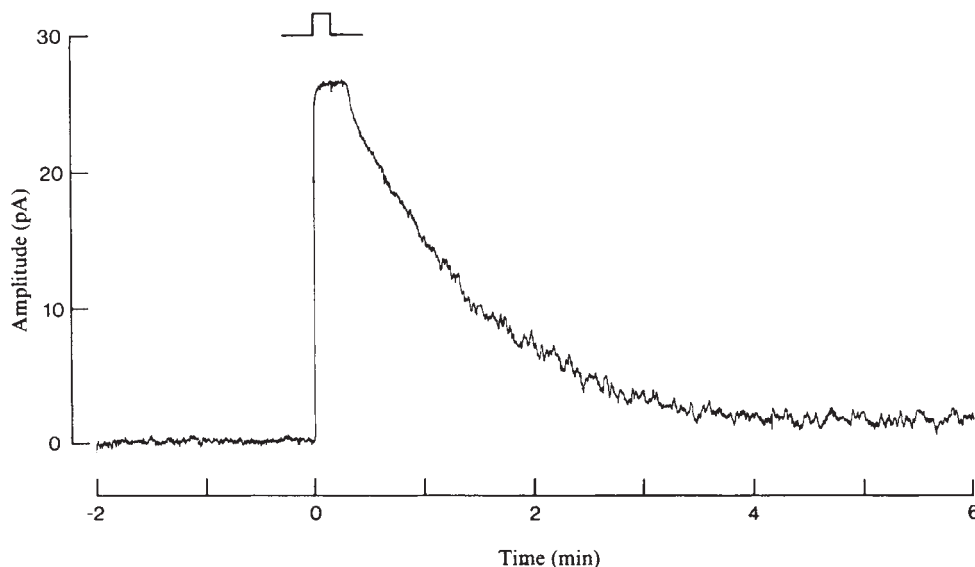
However, the lack of stationarity of the spectral data causes a slight computational difficulty here, as the cell was inevitably recovering during the post-bleach period, and the sensitivity and time course of the flash response changed. Before the lowest record in Fig. 1 the time to peak was 1.5 s, and after the record it was 2.2 s. The averaged response from flashes before and after the fluctuating record is shown in the inset of Fig. 2, and had a time to peak of 1.75 s; it is this averaged response which has been used in computing the expected form of the spectrum. It seems unlikely that this gradual and unavoidable change would significantly affect the form of the predicted spectrum, and the measured fluctuations certainly appear to be explained in terms of spontaneous quantal events.

The rate of equivalent isomerizations may be calculated from the required vertical scaling of the theoretical spectrum. For a random series of identical 'shot' events, the zero frequency asymptote is found from the Fourier transform with $f=0$, and is simply twice the event rate multiplied by the square of the time integral of the shot event (see, for example, ref. 6). For the case of independent activation kinetics this may be shown to reduce to:

$$S(0) = 5.85 \nu t_{\text{peak}}^2 r_{\text{peak}}^2 \quad (2)$$

where $S(0)$ is the zero frequency asymptote, ν s⁻¹ is the rate, and t_{peak} and r_{peak} are the time to peak and peak amplitude of the single photon response respectively. For the cell in Figs 1 and 2, the amplitude of the quantal event in the dark-adapted state averaged about 0.8 pA, but after bleaching the sensitivity was reduced by about 0.12 log units, giving a quantal event amplitude of $r_{\text{peak}}=0.61$ pA. With $t_{\text{peak}}=1.75$ s and $S(0)=3.5 \text{ pA}^2 \text{ Hz}^{-1}$, the rate of events is predicted to be $\nu=3.1$

Fig. 3 Response of another cell to a bleach of about 0.7% (7.2×10^5 photons μm^{-2} , calculated to isomerize 1.8×10^7 rhodopsins). Quantal events before the bleach are not visible as the cell was relatively insensitive. Bicarbonate/ CO_2 -Ringer; 23.6 °C; low pass filtered at 5 Hz.



equivalent isomerizations per s. This is about 2.2 log units greater than the spontaneous rate in the fully dark adapted state (0.02 isomerizations per s, ref. 7).

A similar effect from a cell recorded in bicarbonate/ CO_2 -Ringer is shown in Fig. 3. Before the bleach the baseline was relatively quiet, but during recovery the trace became quite noisy, despite a slight reduction in its flash sensitivity. The power spectrum of these fluctuations was again well fitted by the form predicted from the average post-bleach flash response (not shown). This cell was less sensitive than the previous one and the post-bleach quantal event amplitude was estimated to be $r_{\text{peak}} = 0.12$ pA per isomerization. The zero frequency asymptote $S(0)$ of the spectrum was $0.7 \text{ pA}^2 \text{ Hz}^{-1}$ and substitution in equation (2) gave an estimated mean rate of $\nu = 11$ isomerizations per s for this bleach of 0.7%, about 2.7 log units higher than in absolute dark adaptation.

The conclusion from these results is that, as proposed by Barlow², spontaneous photon-like events occur in the rod outer segments at a much higher rate after a bleach than in absolute dark adaptation. This phenomenon seems to explain qualitatively the existence of the psychophysical equivalent background¹⁴ or dark light^{1,2} which limits visual detection, and the extent to which it may quantitatively account for dark adaptation behaviour is under investigation (D. A. Baylor and T.D.L.). It is interesting that the situation appears similar in invertebrates, where exposure of a dark-adapted locust retina cell to intense light causes an increased rate of occurrence of quantal events¹⁵.

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Light isomerizes the chromophore of bacteriorhodopsin

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The primary photochemical event in the two light-transducing pigments whose chromophore is retinal, rhodopsin or bacteriorhodopsin, is a source of controversy. It was originally proposed that the primary photoevent in the bleaching of rhodopsin is the photoisomerization of the chromophore from 11-*cis* to all-*trans* retinal^{1,2}. Photochemical considerations suggested that a photoisomerization is the primary event in both rhodopsin and bacteriorhodopsin^{3–7}. However, this description of bacteriorhodopsin's photochemistry has been questioned^{8–10}. To elucidate this problem, we determined the isomeric conformation of retinal for two of the photolytic intermediates of bacteriorhodopsin, using a method that enables us to extract chromophores from the photocycle intermediates L and M at low temperatures (–74 °C), and have determined the isomeric conformation of the extracted retinals by HPLC^{11,12}. Here we provide direct evidence that isomerization of the chromophore has taken place in two of the early photocycle intermediates (L and M) of bacteriorhodopsin.

The absorption of a photon by light-adapted bacteriorhodopsin (bR^{LA}), the purple membrane protein of the *Halo-bacterium halobium*, initiates a series of transformations¹³, reminiscent of the bleaching sequence of rhodopsin. The primary photochemical product, K, is a bathochromically shifted species; it decays to the photocycle intermediate L and then to M, which eventually returns to bR^{LA} through the intermediates N and O. bR^{LA} slowly converts to the dark-adapted bacteriorhodopsin (bR^{DA}). It has been determined that the isomeric form of chromophore of bR^{LA} is almost completely all-*trans* retinal, whereas bR^{DA} has a mixture of 13-*cis* and all-*trans* retinals with a ratio of 1:1. The isomeric compositions were

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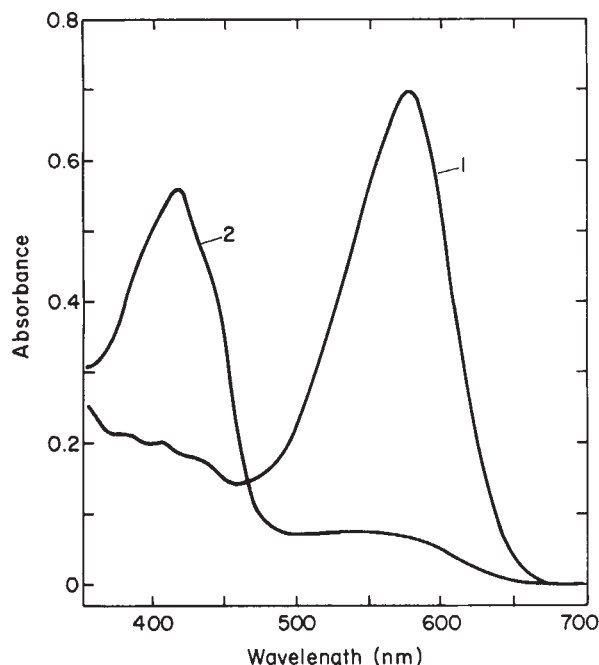


Fig. 1 Photochemical conversion of bR^{LA} to M at -74°C . Curve 1, bR^{LA} in a 25% NaCl solution adjusted to pH 9.5 (0.1 M carbonate buffer) and mixed with glycerol (1:2) at -74°C . Curve 2, the photosteady-state mixture produced by irradiating with $\lambda > 580\text{ nm}$ light (Corning filter 2-73) for 15 min at -74°C . The sample was irradiated with the light from a 200-W tungsten lamp using a glass filter. The light was routed to the sample cell through an optical glass fibre (5-mm diameter).

originally determined by chromophore extraction methods^{11,14,15}. The validity of determining the isomeric composition by the extraction technique was later verified by several non-destructive spectroscopic techniques¹⁶⁻¹⁹.

To extract chromophores from the L and M intermediates at low temperatures, the extraction solvent should not freeze at low temperatures (-74°C), it should denature the pigments at the low temperature, and the retinal should not be isomerized by the solvent itself. We found an extraction solvent composed of buffer/glycerol/dichloromethane/hexane (1.6:3.3:1:4) which satisfied the above conditions. The solvent was first emulsified by sonication (30 s). Then a syringe cylinder with the tip blocked by a Teflon plug, containing the bR sample in 67% glycerol was inserted into a test tube containing the solvent. This in turn was immersed in a dry ice-ethanol bath (-74°C) and the bR irradiated in the syringe with an optical light guide. The plunger was then inserted into the syringe cylinder and the irradiated bR injected into the extraction solvent. Next, the bR sample was mixed thoroughly with the extraction solvent, using a precooled (-74°C) spatula. The temperature of the sample was monitored with a thermocouple. This procedure denatured the irradiated bR sample at -74°C as shown by the total fading of the colour to pale yellow. The mixture was then warmed to 0°C and chromophores extracted by sonication. Extraction by the sonication method gave a substantially greater yield ($60 \pm 20\%$) than the syringe method ($<10\%$) and exhibited no preferential extraction of either isomer.

To insure that the isomeric composition of the extracted retinals represented that of the intact pigment and that no isomerization had taken place during our extraction procedure, we extracted the chromophores of bR^{LA} and bR^{DA} . Table 1 shows that isomerization of bR^{LA} and bR^{DA} extracted in our conditions are basically the same as the compositions previously reported. The pH, glycerol and temperature we used affected the isomeric composition of the chromophore slightly.

bR^{LA} suspended in a 25% NaCl solution adjusted to pH 9.5 was mixed with glycerol (1:2) to prevent crystallization at low temperatures. The extraction of retinals from bR^{LA} at 0°C yielded $94 \pm 3\%$ all-*trans* retinal and $6 \pm 3\%$ 13-*cis* retinal. When bR^{LA} was cooled to -74°C (Fig. 1, curve 1) and irradiated with light of wavelengths greater than 580 nm ($\lambda > 580\text{ nm}$ light), the M intermediate was formed (Fig. 1, curve 2). In the dark, the absorption spectrum was stable. Extraction of the chromophores from this preparation yielded $14 \pm 3\%$ all-*trans* retinal and $86 \pm 3\%$ 13-*cis* retinal. The amount of M, based on the decrease in absorption at 570 nm, was estimated to be 91% (Fig. 1, curve 2). Therefore, our results suggested that the isomeric form of retinal in M was at least 95% 13-*cis* retinal. After making the M intermediate at -74°C , the sample was warmed to 0°C , and after waiting for 10 min to ensure that M had returned to bR^{LA} , the chromophores were extracted. This preparation yielded $93 \pm 4\%$ all-*trans* retinal and $7 \pm 4\%$ 13-*cis* retinal, which corresponds to the composition in bR^{LA} before making M. These results suggested that light isomerized the chromophore in the conversion of bR^{LA} to M and the chromophore was thermally re-isomerized in returning from M to bR^{LA} in the dark.

The intermediate L, which is the precursor of M, is produced as a member of a photosteady-state mixture containing L, M and bR^{LA} when bR^{LA} (in a 25% NaCl solution adjusted to pH 7.2) is irradiated with $\lambda > 630\text{ nm}$ light as shown in Fig. 2, curve 2. Table 1 shows that before making L, bR^{LA} in glycerol contains $92 \pm 2\%$ all-*trans* retinal and $8 \pm 2\%$ 13-*cis* retinal; after making L and warming the sample to 0°C for 10 min it contains $92 \pm 2\%$ all-*trans* retinal and $8 \pm 2\%$ 13-*cis* retinal. Denaturation of the photosteady state formed at -74°C and extraction of its chromophores yields $53 \pm 4\%$ all-*trans* retinal and $47 \pm 4\%$ 13-*cis* retinal. The content of L, M, and bR^{LA} in this preparation is difficult to determine precisely because two different extinction coefficients for L have been proposed^{20,21}. The amounts of L, M and bR^{LA} in the mixture shown in spectrum 2 of Fig. 1 are calculated to the 57%, 13% and 30%, respectively, assuming $\epsilon_L = 61,000$ (ref. 20) (without consideration of bypass of $L \rightarrow bR^{LA}$) and as 29%, 13% and 58%, respectively, assuming

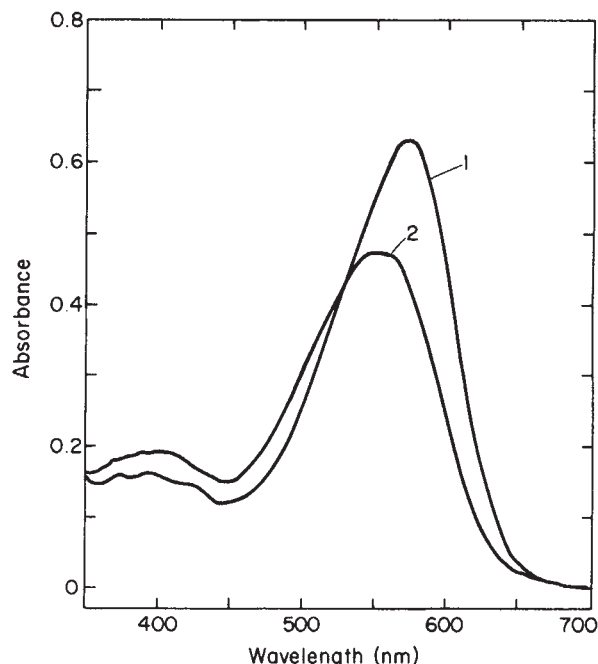


Fig. 2 Photochemical conversion of bR^{LA} to L at -74°C . Curve 1, bR^{LA} in a 25% NaCl solution adjusted to pH 7.2 (0.1 M imidazole-HCl) and mixed with glycerol (1:2) at -74°C . Curve 2, the photosteady-state mixture produced by irradiating with $\lambda > 630\text{ nm}$ light (Corning filter 2-59) for 15 min at -74°C . Irradiation of sample was carried out as shown in Fig. 1.

Table 1 Retinal isomers extracted from bR^{LA}, bR^{DA}, M and L

Species	Conditions	% Composition 13- <i>cis</i>	% Composition All- <i>trans</i>	Yield (%)
a pH 9.5, 25% NaCl				
bR ^D		55 ± 2	45 ± 2	43 ± 13
bR ^{DA} (glycerol)		54 ± 2	46 ± 2	45 ± 9
bR ^{DA} (glycerol)	denatured at -74 °C and extracted at 0 °C	54 ± 3	45 ± 3	56 ± 4
bR ^{LA}		6 ± 3	94 ± 3	57 ± 8
bR ^{LA} (glycerol)		7 ± 4	93 ± 4	45 ± 12
bR ^{LA} (glycerol)	denatured at -74 °C and extracted at 0 °C	11 ± 3	89 ± 3	55 ± 9
M (glycerol)	bR ^{LA} irradiated with λ > 580 nm light for 15 min, denatured at -74 °C and extracted at 0 °C	86 ± 3	14 ± 3	55 ± 15
bR ^{LA} (glycerol)	M was warmed up to 0 °C and kept 10 min at 0 °C	7 ± 4	93 ± 4	46 ± 8
b pH 7.2, 25% NaCl				
bR ^{DA}		52 ± 2	48 ± 2	59 ± 8
bR ^{DA} (glycerol)		54 ± 2	46 ± 2	65 ± 20
bR ^{DA} (glycerol)	denatured at -74 °C and extracted at 0 °C	56 ± 2	44 ± 2	70 ± 15
bR ^{LA}		3 ± 2	97 ± 2	56 ± 10
bR ^{LA} (glycerol)		8 ± 2	92 ± 2	53 ± 10
bR ^{LA} (glycerol)	denatured at -74 °C and extracted at 0 °C	11 ± 2	89 ± 2	60 ± 8
L	bR ^{LA} irradiated with λ > 630 nm light for 15 min, denatured at -74 °C and extracted at 0 °C	47 ± 4	53 ± 4	52 ± 10
bR ^{LA} (glycerol)	L was warmed up to 0 °C and kept for 10 min at 0 °C	8 ± 2	92 ± 2	60 ± 12

Each experiment was repeated at least five times. Dark adaptation (bR^{DA}) was assured by allowing the bR sample to stand overnight at room temperature for each set of conditions (pH, glycerol). Light adaptation (bR^L) was accomplished by irradiating the sample with a slide projector for 20 min at 0 °C, followed by immediate extraction. Denaturation and extraction were performed at 0 °C unless otherwise described. An attempt to quantify the efficiency of retinal extraction was made by measuring the A₃₆₅ in hexane solution after extraction. The retinal concentrations in the solution were determined by the retinal composition and using the following extinction coefficients: (4.58 × 10⁴ for all-*trans*, 3.84 × 10⁴ for 13-*cis* at 365 nm)²⁴. Separation and analysis of retinals in the extract were carried out by HPLC with micro-Porasil column (Waters Associates). The sample was eluted at a constant flow rate (2 ml min⁻¹) with 4% ether-hexane (v/v). Two well resolved peaks were detected spectrophotometrically (λ = 365 nm). They were identified as the 13-*cis* and all-*trans* isomers of retinal by a comparison of their retention times with those of authentic samples.

$\epsilon_L = 49,000$ (ref. 21) (with consideration of bypass of L → bR^{LA}). Taking bR^{LA} as all-*trans* and M as 13-*cis* (see above) there is a reasonable correlation between the per cent L and M present spectroscopically from (42–70%) and the per cent of the 13-*cis* isomer present (47 ± 4%) if we assume that chromophore of L is 100% 13-*cis* retinal.

Thus, the present results suggest that light has initiated an event which results in the chromophore of bR^{LA} being isomerized from the all-*trans* to 13-*cis* conformation by the time of the appearance of the L intermediate, and that the 13-*cis* chromophore is thermally re-isomerized to the all-*trans* form in going from M to bR^{LA} in the dark. By far the simplest explanation of our results is the general hypothesis, based on photochemical studies by Rosenfeld *et al.*³ and Hurley *et al.*⁴, that the primary event in bacteriorhodopsin is a photoisomerization requiring that the isomeric conformation of retinal in bR^{LA} be different from its conformation in L and M. Some indications of an isomerization step have also been noticed on the basis of resonance Raman measurements^{22,23} although there is still controversy about the interpretation of these spectra.

Our results may be compared with the work of Pettei *et al.*¹¹, who first suggested the isomerization event was from the all-*trans* to the 13-*cis* isomer. These authors extracted chromophores from 'M-like intermediates' in ether/high salt preparations and guanidinium HCl/high pH preparations at 0 °C and concluded that M yields the 13-*cis* isomer, although the

correlation between M and the 13-*cis* isomer of the former preparation was rather poor. We find a quite good correlation between the amount of 13-*cis* retinal present and the amount of the M intermediate present. It is possible that the nature of M in ether/high salt and guanidinium HCl/high pH at 0 °C is different from M at normal conditions. More likely, as Pettei *et al.* did not actually measure the absorption spectra of their solutions, they had not achieved full photoconversion of bR to M.

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Leukaemia virus infection promotes fibroblast transformation by normal BALB/c mouse DNA

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All normal cells are thought to carry genetic information for oncogenic transformation, which, on activation to continuous expression, might make the cell cancerous¹. The presently known transforming retroviruses contain transforming genes which were probably derived by recombination of a slow oncogenic retrovirus with cellular sequences closely related to these genes². It was recently reported³ that cellular DNA fragments from normal tissue culture cells could transform mouse fibroblasts *in vitro* with a low efficiency. High efficiency of transformation was observed in secondary transfections only when high molecular weight DNA from transformed recipient cells was used as the transforming agent³. We observed that DNA isolated from different BALB/c mouse organs can transform both NIH/3T3 and BALB/3T3 cells, although at a low frequency⁴. In attempts to increase the initial efficiency of transformation, we have found that preinfection of recipient 3T3 cells with murine leukaemia viruses markedly enhances focus formation by normal BALB/c DNA fragments.

Table 1 Effect of leukaemia virus preinfection on early transformation of NIH/3T3 fibroblasts with normal BALB/c DNA

Recipient cells	Treatment	Fraction of transformed cultures*	Average no. of foci per dish*	Average frequency of transformation ($\times 10^{-6}$ per μg DNA per cell)
NIH/3T3	BALB/c DNA	0/33	0	0
	<i>Escherichia coli</i> DNA	0/33	0	0
	Transfection buffer	0/33	0	0
NIH/3T3/RMuLV ⁺ (Rauscher LV)	BALB/c DNA	3/3	4	2
	<i>E. coli</i> DNA	0/3	0	0
	Transfection buffer	0/3	0	0
NIH/3T3/MoMuLV [‡] (Moloney LV)	BALB/c DNA	33/33	18	7
	<i>E. coli</i> DNA	0/33	0	0
	Transfection buffer	0/33	0	0

DNA was isolated from liver, spleen and thymus of 5-week-old germ-free BALB/c mice as previously described¹⁰. DNA solutions were exhaustively dialysed against $0.1 \times \text{SSC}$ (0.015 M NaCl , $0.0015 \text{ M trisodium citrate}$, $\text{pH } 7.0$) before use. The transfection experiments were performed by a modification of the Graham and van der Eb method¹¹. DNA was dissolved in transfection buffer ($\text{pH } 6.95$) to a concentration of $25 \mu\text{g ml}^{-1}$, sheared by passing the solutions 20 times through a 0.8-mm needle to a molecular weight of about 10×10^6 , as determined by agarose gel electrophoresis, and precipitated with 2.5 M CaCl_2 to a final concentration of 125 mM ; 0.5 ml of DNA precipitate was added to 2.5 ml of fresh medium on dishes of 3T3 cells seeded 20 h earlier at a concentration of 3×10^5 cells per 60 mm dish. Eighteen hours after the addition of DNA, the cells were trypsinized. Focus formation was evaluated 12–15 days after the DNA treatment.

* Average values from one (NIH/3T3/RMuLV) to four (NIH/3T3/MoMuLV) experiments.

[†] NIH/3T3 cells were infected with Rauscher leukaemia virus four days before the transfection experiment.

[‡] NIH/3T3/MoMuLV is a clonal isolate of NIH/3T3 cells productively infected with Moloney leukaemia virus (gift of Dr H. P. J. Bloemers).

To increase the efficiency of transformation of recipient cells by normal BALB/c DNA fragments, we manipulated the DNA-treated cells using procedures reported to heighten the expression of newly acquired DNA in recipient cells. This involved adjuvant treatment with dimethyl sulphoxide (DMSO) or glycerol^{5,6} or the cultivation of DNA-treated cells in the presence of polybrene, insulin and hydrocortisone⁷. However, with none of these procedures did we observe the focus formation directly on DNA-treated cells or soon after the DNA treatment. Instead, DMSO treatment and cultivation of the DNA-treated cells in the presence of polybrene, hydrocortisone and insulin had a toxic effect on the 3T3 cells.

We then investigated whether infection of recipient cells with non-transforming leukaemia viruses had any positive effect, as it has been reported that a similar procedure had a promoting effect on chemical carcinogenesis *in vitro*⁸. The recipient NIH/3T3 cells were productively infected with either Moloney

(MoMuLV) or Rauscher (RMuLV) leukaemia virus. The morphology of the 3T3 cells infected with these leukaemia viruses was indistinguishable from that of uninfected controls. The transformation efficiency was greatly enhanced by this procedure. On both MoMuLV- and RMuLV-infected NIH/3T3 fibroblasts, focus formation could be scored as early as 12–15 days after exposure to BALB/c DNA fragments in the cells of the first passage after the DNA treatment (Table 1). In preliminary experiments, supernatants from these transformed cultures showed no transforming activity.

The leukaemia virus-infected fibroblasts are transformed by single-hit kinetics (Fig. 1). This indicates that, with the promoting effect of a viral gene, focus formation of fibroblasts is induced by a single BALB/c gene which could be any of the various transforming genes supposed to be present in the mouse genome. The frequency of transformation reached a value of $0.5\text{--}1$ focus forming units per μg of DNA, which is comparable to that observed for transformation induced with proviral DNA fragments (molecular weight 10×10^6) isolated from Abelson virus-transformed lymphoma cells (V. K.-K. and K. J. B., in preparation).

The nature of the observed enhancement of fibroblast transformation by normal BALB/c DNA fragments after leukaemia virus infection is not clear. The leukaemia virus might provide complementary functions required for expression of endogenous mouse transforming genes⁹ such as promoter sites.

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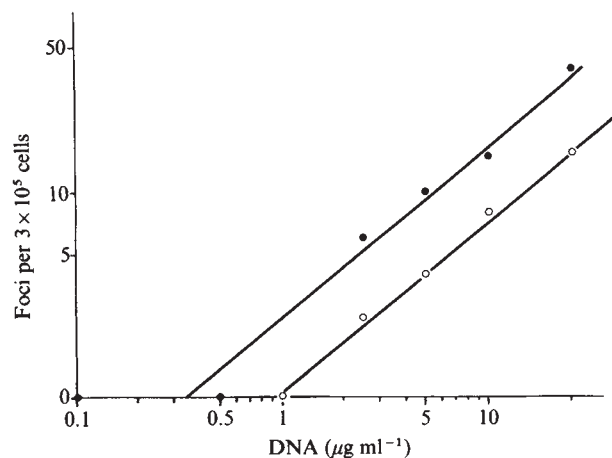


Fig. 1 Kinetics of transformation of NIH/3T3 cells productively infected with Moloney leukaemia virus by fragments of normal BALB/c DNA. The total amount of DNA in each DNA inoculum was adjusted to $25 \mu\text{g ml}^{-1}$ by the addition of calf thymus DNA before calcium precipitation. ● and ○ represent results of two independent experiments. Three recipient cultures were treated with each dose of DNA. Foci were scored 12–15 days after the treatment with DNA fragments.

Inhibition of 40S-Met-tRNA^{Met}_i ribosomal initiation complex formation by vaccinia virus

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Infection with vaccinia virus (a poxvirus) quickly^{1,2} and efficiently shuts off host protein synthesis in the presence of actinomycin D (refs 3-5) or cycloheximide⁶⁻⁸. The cellular messenger RNA apparently remains stable in the infected cells exposed to inhibitors of viral gene transcription^{5,9,10}. In some cases vaccinia viral RNA or poly(A) synthesis have been implicated in the establishment of this effect^{5,7,11}. However, in the presence of cordycepin (3-deoxyadenosine) which blocks viral gene transcription¹² and cytoplasmic poly(A) synthesis⁹, cellular protein synthesis is still efficiently inhibited in vaccinia virus-infected cells^{9,12}. This shutoff is also observed *in vitro*, in the corresponding cell-free extracts¹³, and in a reticulocyte lysate¹⁴⁻¹⁶. Therefore the shutoff of host protein synthesis is probably mediated by a factor associated with vaccinia virions. We now report that the formation of the 40S-Met-tRNA^{Met}_i initiation complex is inhibited in cytoplasmic extracts derived from vaccinia virus-infected cells exposed to cordycepin to block viral gene expression. A similar inhibition is found in reticulocyte lysates incubated with purified vaccinia cores, confirming the hypothesis that the factor associated with the viral cores is responsible for the inhibition observed in vaccinia virus-infected cells exposed to inhibitors of transcription.

The binding of initiator Met-tRNA^{Met}_i to 40S ribosomal subunits was measured as previously described¹⁷ after adding either ³⁵S-methionine or purified ³⁵S-Met-tRNA^{Met}_i to cytoplasmic extracts derived from vaccinia virus-infected or uninfected cells. The extracts were pre-incubated with sparsomycin to block the elongation step. Figure 1 shows that 40S-Met-tRNA^{Met}_i initiation complex formation was inhibited to 60% in the infected cell extracts. A similar inhibition was observed when using unwashed ribosomes and post-ribosomal supernatant fractions (S100) instead of the corresponding infected cell extracts; moreover the inhibition was associated with the unwashed ribosomes from the infected cells (data not shown). This reduced amount of ³⁵S-methionine associated with 40S ribosomes indeed represented a reduced formation (or increased dissociation) of the 40S-Met-tRNA^{Met}_i initiation complex as proved by the following results: (1) increasing the incubation time after the addition of ³⁵S-methionine led to a plateau value of 40S-Met-tRNA^{Met}_i complex formation after 30-45 min for control and infected cell extracts; this plateau was 50% lower in the infected cell extracts (data not shown). It is worth noting that the time needed to reach equilibration was much longer in the Ehrlich ascites tumour (EAT) cell-free system than previously observed in the reticulocyte lysate¹⁷. (2) The addition of purified ³⁵S-Met-tRNA^{Met}_i instead of ³⁵S-methionine to the cytoplasmic extracts from vaccinia virus-infected cells exposed to cordycepin, resulted in a similar inhibition (50-60%) of 40S-Met-tRNA^{Met}_i initiation complex formation. Figure 2a shows that the amount of free charged tRNA decreased identically in control and infected cell extracts, probably as a result of degradation or deacylation. Figure 2b reveals a plateau value of the ³⁵S-Met-tRNA^{Met}_i bound to the 40S subunit (expressed in % of residual labelled initiator tRNA), reached within 5-10 min for both extracts. This value was 50% lower in the infected cell extract.

The following arguments indicate that this decrease in 40S-Met-tRNA^{Met}_i complex formation is likely to account for the block in initiation of polypeptide chains previously observed in the vaccinia virus-infected cell extracts¹³: (1) the rate of protein synthesis *in vitro* in vaccinia virus-infected cell extracts is inhibited¹³ to a similar extent (50-70%) to that of binding of ³⁵S-Met-tRNA^{Met}_i to 40S ribosomes (Fig. 2, Table 1); (2) the inhibition of the 40S-Met-tRNA^{Met}_i complex formation in the infected cell extract was also observed when the corresponding ribosomes and S100 fraction were incubated as described in Fig. 1 but in the absence of sparsomycin; these conditions allow protein synthesis to occur and the ³⁵S-radioactivity associated with the more rapidly sedimenting ribosomal particles was also strongly reduced in the infected cell extract (data not shown); (3) the addition of ribosomal salt wash (RSW) from uninfected cells stimulated three- to five-fold the amount of labelled initiator tRNA bound to 40S particles in the control and infected cell extracts but did not overcome the inhibition of the infected cell

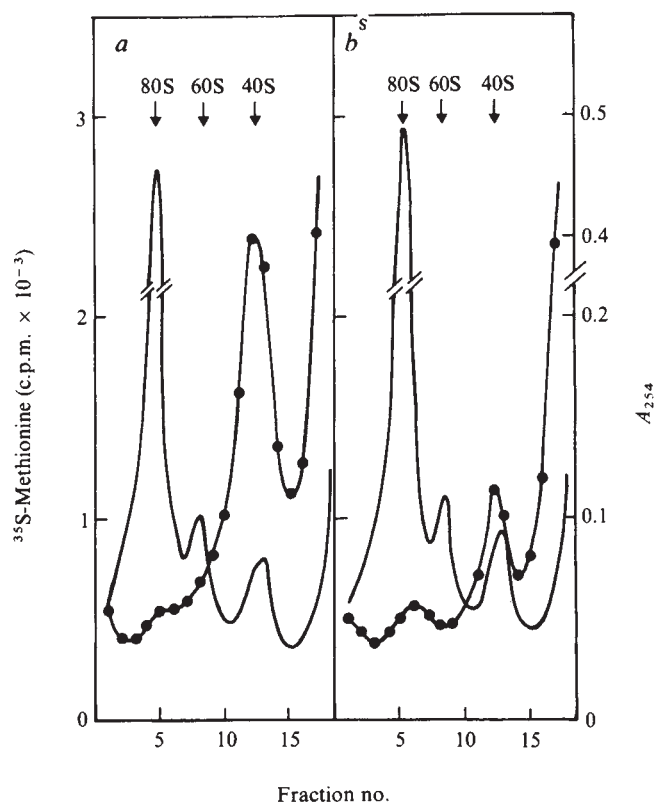


Fig. 1 Binding of ³⁵S-Met-tRNA^{Met}_i to 40S ribosomal subunits in the cytoplasmic extracts from uninfected (a) and vaccinia virus-infected (b) cells exposed to cordycepin. The lysates prepared from EAT¹³ were diluted to 50 A₂₆₀ units ml⁻¹ in 3.5 mM Mg acetate, 0.1 M K acetate and all the other components of standard protein synthesis *in vitro*¹³. Incubation (50 µl samples) was at 30 °C for 5 min in the presence of 0.3 mM sparsomycin. Then ³⁵S-methionine was added (800 µCi ml⁻¹) and after a further 10 min incubation, the reaction mixture was diluted with 5 vols cold buffer H: 20 mM HEPES-KOH, pH 7.5, 20 mM KCl, 2 mM Mg acetate, 0.1 mM EDTA and centrifuged through 15-30% (w/v) sucrose gradients prepared in buffer H, for 100 min at 49,000 r.p.m. in a Beckman SW 50.1 rotor at 4 °C. The gradients were scanned at 254 nm with an Isco monitor (5 mm path) and collected into 23 fractions. ³⁵S-Met-tRNA (●—●) was measured after cetyltrimethylammonium bromide (CTAB) precipitation¹⁷ and filtration on Whatmann GF/C. The amount of CTAB-precipitable radioactivity was similar for control and infected cell extracts in the top fractions (not shown). About 0.35 molecules of charged initiator tRNA was bound per 40S ribosome particle in the control extract, as estimated from the values of the endogenous methionine pool¹³ and from the determination of the free 40S ribosomes based on the optical scans. (Trace of A₂₅₄ shown as continuous lines.)

extracts (Table 1). This corroborates a similar effect of these additions on protein synthesis in the corresponding extracts¹³.

As protein synthesis is also inhibited at the level of initiation in the reticulocyte lysates incubated with vaccinia cores¹⁴, it was important to measure the 40S-Met-tRNA_i^{Met} initiation complex formation in this *in vitro* system to demonstrate a similar mechanism for the viral core-mediated inhibition. Figure 3 shows that the binding of endogenously labelled ³⁵S-Met-tRNA_i^{Met} to 40S ribosomes is strongly inhibited when the reticulocyte lysate is incubated with purified viral cores. A similar inhibition was observed when adding purified ³⁵S-Met-tRNA_i^{Met} to the reticulocyte lysate preincubated with the vaccinia cores (data not shown). When the concentration of viral cores was varied, the inhibition of 40S initiation complex formation was equal to the inhibition of protein synthesis: adding 3 A₂₆₀ units per ml of cores caused 42% inhibition of 40S-Met-tRNA_i^{Met} complex and 49% inhibition of *in vitro* protein synthesis, whereas adding 9 A₂₆₀ units per ml of cores induced 65 and 66% inhibition respectively. This result confirms the hypothesis whereby the reduced binding of initiator Met-tRNA to 40S ribosomes is indeed responsible for the inhibition of initiation of protein synthesis previously observed¹⁴.

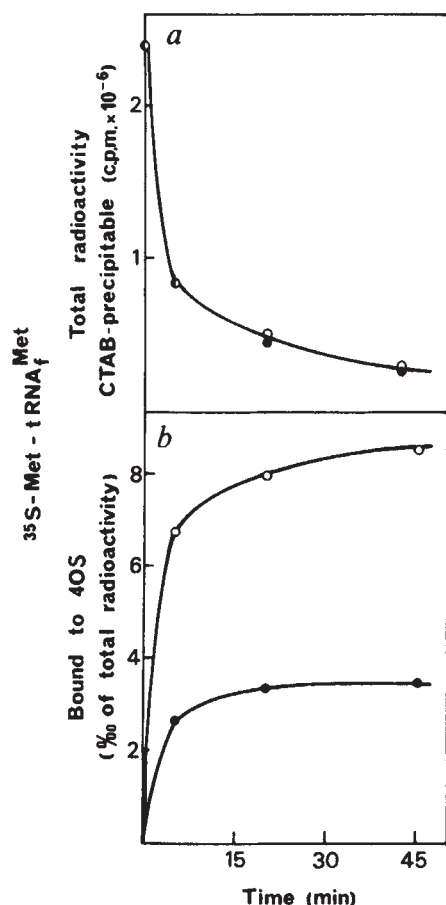


Fig. 2 Kinetics of ³⁵S-Met-tRNA_i^{Met} binding to 40S ribosomal subunits from ribosomes of uninfected and vaccinia virus-infected cells exposed to cordycepin. Ribosomes and supernatant (S100) fractions were prepared by centrifuging cytoplasmic extracts¹³ at 198,000g for 1 h. The ribosomal pellet was suspended in 10 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM Mg acetate, 2.5 mM dithiothreitol. The S100 fraction and ribosomes were stored in liquid nitrogen. Ribosomes (40 A₂₆₀ units ml⁻¹) and S100 fraction (7.5 A₂₆₀ units ml⁻¹) were incubated and analysed on sucrose gradients as described in Fig. 1 except that purified ³⁵S-Met-tRNA_i^{Met} (2.4 × 10⁶ c.p.m., 30 μg ml⁻¹) was added instead of ³⁵S-methionine. The time of incubation is indicated on the abscissa. Total amount of radioactivity was calculated from the summation of fractions from each gradient. Ribosomes and S100 were from control (○) or infected (●) cells.

Table 1 Effect of RSW addition on the formation of the 40S-Met-tRNA_i^{Met} complex

Experiment	Extract from cells	Ribosome concentration (mg ml ⁻¹)	³⁵ S-Met-tRNA _i ^{Met} bound to 40S			
			-RSW		+RSW	
			Radio-activity (c.p.m.)	Inhibition (%)	Radio-activity (c.p.m.)	Inhibition (%)
I	Control	2.7	13,000		42,000	
	Infected	2.9	5,500	58	22,000	47
II	Control	1.4	3,700		16,800	
	Infected	1.5	1,700	54	9,100	46

The binding of ³⁵S-Met-tRNA_i^{Met} to 40S subunits was measured as described in Fig. 2. RSW free of 40S subunits was prepared from uninfected cells¹³ and used at 1 mg protein ml⁻¹. Mg acetate was added at 2 mM together with 50 μM spermine when RSW was present¹³. The addition of RSW induces an increase of 38% (expt I) or 57% (expt II) in the amount of native 40S subunits, as measured from the optical scans.

The results presented here reveal that the shutoff of host protein synthesis mediated by vaccinia virus (in the absence of viral gene expression) occurs at the level of Met-tRNA_i^{Met} binding to 40S ribosomes both in reticulocyte lysate incubated in the presence of vaccinia cores and in lysates from vaccinia virus-infected cells exposed to cordycepin. The fact that the same site of lesion is found in both cases strongly suggests a similar mechanism of inhibition. This inhibition in initiation of protein synthesis is generally believed to occur before the binding of mRNA and therefore would result in an inhibition of both cellular and viral mRNA translations. This agrees with previous studies which demonstrated a nonspecific inhibition of cellular and early viral mRNA translation in some experimental conditions^{6,10,18}. Presumably a virus-coded protein, synthesized very early in the infection, is involved in releasing the translational block associated with the input virions^{6,10,18}.

The formation of the 40S-Met-tRNA_i^{Met} complex appears to be an important target for the translational regulation in different physiological systems¹⁹⁻²² and in most cases, the corresponding inhibition is released by the addition of ribosomal salt wash or purified eIF-2 (refs 23, 24). Although the addition of ribosomal salt wash from uninfected cells stimulated the binding of Met-tRNA_i^{Met} to 40S subunits from infected cells, and also protein synthesis in these extracts¹³, it did not abolish the difference between control and infected cells, suggesting the existence of a lesion which may lie in the ribosomes themselves rather than in the initiation factors. This conclusion is reinforced by the finding that addition of purified eIF-2 to reticulocyte lysates inhibited by vaccinia cores produced at best only a partial stimulation of protein synthesis (ref. 14 and R. Kaempfer and E. Katz, personal communication), confirmed the inhibition of protein synthesis by vaccinia cores and did not detect reversal by the addition of 2-aminopurine or purified eIF-2. We must conclude that although the inhibition caused by vaccinia infection superficially resembles the inhibition seen in reticulocyte lysates incubated with double-stranded RNA or in the absence of haemin, where the impairment of 40S-Met-tRNA_i^{Met} complex formation is believed to result from alterations to eIF-2, it must have a different cause. Apparently another mechanism to regulate the level of 40S-Met-tRNA_i^{Met} complexes must exist. The identification of the inhibitor associated with vaccinia cores, now being carried out in the laboratory, may help to elucidate the mechanism of inhibition. A possible role for a vaccinia structural protein in the establishment of the shutoff of host protein synthesis has previously been suggested²⁵.

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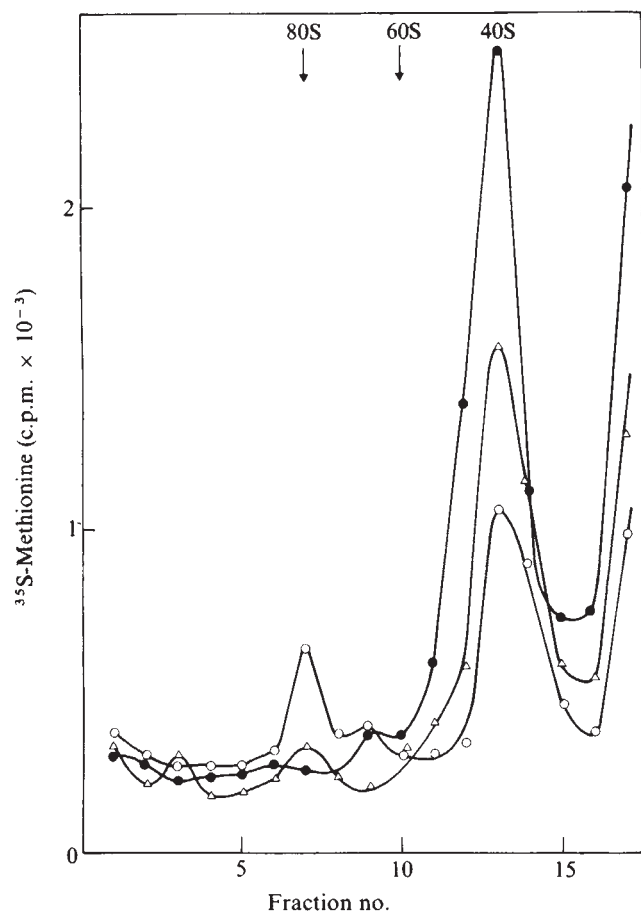


Fig. 3 Inhibition of 40S-Met-tRNA^{Met} complex formation by purified vaccinia virus cores. First the reticulocyte lysate (20 μ l) was preincubated at 25 $^{\circ}$ C for 10 min with vaccinia cores (Δ , 3A₂₆₀ units ml⁻¹; 0, g A₂₆₀ units ml⁻¹) or without cores ($\bullet$) in the presence of 0.33 mM sparsomycin in a total volume of 50 μ l of protein synthesis reaction mixture¹⁴. Then 5 μ l of ³⁵S-methionine were added (839 Ci mol⁻¹, 43 μ Ci) and after a further incubation of 5 min at 30 $^{\circ}$ C the reaction mixtures were analysed as described in Fig. 1, except for the centrifugation step which was carried out in a Beckman SW 41 rotor for 220 min at 40,000 r.p.m. The amount of CTAB-precipitable radioactivity in the top fractions was similar in the control and in the presence of the cores (not shown).

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S-adenosylmethionine—a novel regulator of aspartate kinase

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Man derives 70% of his dietary requirement of protein directly from the grains of cereals and legumes. These sources are respectively deficient in lysine (and secondarily threonine) and methionine and much effort is being devoted to their improvement¹. All three amino acids are derived from aspartate via a common metabolic pathway (Fig. 1) in which the first reaction catalysed by aspartate kinase is a key regulatory step limiting their production. In microorganisms, regulation of aspartate kinase occurs by a variety of mechanisms, commonly involving feedback inhibition of one or more isoenzymes by Lys plus Thr, Lys alone or Thr alone². On the other hand, Met control of this step does not seem to conform to a general pattern. Met represses, but does not inhibit aspartate kinase II of *Escherichia coli*; in other species Met can enhance or modify the effects of Lys or Thr³. Similarly, varied controls involving Lys and Thr have been reported for the enzymes from higher plants⁴ with only one report of an effect of Met⁵. In contrast to these previous results, we suggest here that the methionine derivative (*S*)-S-adenosyl-L-methionine (AdoMet) is an important regulator of the Lys-sensitive aspartate kinase of higher plants, and that this regulatory mechanism is highly conserved. There is thus a major synergistic interaction of the two nutritionally deficient amino acids Lys and Met to inhibit their own syntheses at the primary regulatory step in the pathway.

Initial studies suggested that plant aspartate kinases may be inhibited by Lys alone^{6,7}, Thr alone⁸ or in a concerted manner by Lys plus Thr⁹. More recent work has shown that certain plants contain two isoenzymes, one sensitive to Thr and one to Lys, the relative proportions of which may vary at different stages of plant development¹⁰⁻¹². Thr also regulates the activity of homoserine dehydrogenase⁴ (Fig. 1(3)). So far the only effect of Met that has been reported in detail is a concerted effect with Lys in which barley aspartate kinase was inhibited 35% by 0.5 mM Lys and 70% by 0.5 mM Lys plus 0.5 mM Met; Met had no effect on its own⁵. Evidence that Met, or a product derived from it, inhibits Met synthesis *in vivo* has been obtained from feeding studies with [³⁵S]O₂⁴⁻ in *Lemna paucicostata*¹³ and barley (S. W. J. Bright, personal communication). Recently it has been found^{14,15} that AdoMet regulates the latter part of the pathway by strongly activating Thr synthase (Fig. 1(4)), implying that Thr synthesis may occur only when the cell has made sufficient Met to satisfy its needs for AdoMet. AdoMet has a very important role in plant metabolism, being the predominant biological methyl donor in methyl transfer reactions and a precursor of polyamines and the plant hormone ethylene¹³. AdoMet is rapidly synthesized if Met is fed to intact plants^{13,16}. It may thus be that AdoMet rather than Met itself acts as a regulator of Met synthesis. We have therefore investigated its effect on aspartate kinase from a range of higher plants.

Lys-sensitive aspartate kinase was partially purified from barley and muskmelon seedlings, pea and maize leaves and developing seeds of pea and field bean. The effect of various concentrations of Lys and AdoMet on its activity was tested. AdoMet on its own had little effect at concentrations up to 0.4 mM but Lys inhibited the activity from all species studied.

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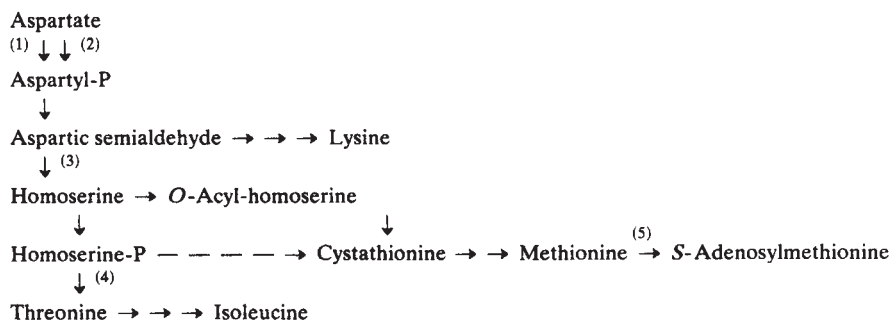


Fig. 1 Diagram of the biosynthetic pathway for the aspartate family of amino acids. Enzymes: (1) and (2) lysine-sensitive and threonine-sensitive aspartate kinases (EC 2.7.2.4); (3) homoserine dehydrogenase (EC 1.1.1.3); (4) threonine synthase (EC 4.2.99.2); (5) methionine adenosyltransferase (EC 2.5.1.6). The pathway in higher plants (→) differs from that in microorganisms (— — —) which synthesize cystathionine via O-acetyl- or O-succinyl-homoserine rather than O-phosphohomoserine.

However, when equal amounts of AdoMet and Lys were added together, a marked synergistic inhibition was observed, with the strongest effect on the barley enzyme (Fig. 2). The AdoMet used was about 90% pure and contained small amounts of S-adenosylhomocysteine, 5'-methylthioadenosine and homoserine as detected by TLC. However, inhibition was due to AdoMet since: (1) hydrolysis of the product at pH 3 (100 °C, 30 min), which completely breaks down AdoMet to 5'-methylthioadenosine and homoserine¹⁷, reduced the inhibition (by 0.25 mM AdoMet or hydrolysis products) in the presence of 25 µM Lys from 60% to 19%; and (2) S-adenosylhomocysteine at 0.25 mM together with 25 µM Lys gave only 9% inhibition. Thr (5 mM) also inhibited part of the isolated aspartate kinase activity from pea leaves (25%) and field bean and pea seed (10%); Thr-sensitive activity was always additive to the (Lys + AdoMet)-sensitive activity. When AdoMet was added to a separate preparation from cotyledons of 4-day-old pea seedlings, which was 90% inhibited by Thr and insensitive to Lys⁸, there was no effect on activity either in the presence or absence of Thr. We therefore conclude that AdoMet acts only on the Lys-sensitive aspartate kinase. It is possible that the previously reported effect of Met plus Lys on the barley enzyme⁵ was due to the presence of methionine adenosyltransferase (Fig. 1(5)) in the preparation and all the required substrates for AdoMet formation in the assay tube.

Although aspartate kinase can be completely inhibited by Lys alone, the presence of AdoMet greatly enhances its sensitivity toward Lys, decreasing the concentration of Lys required to

cause half-maximal inhibition severalfold (Table 1). Conversely, when small amounts of Lys are present, the activity of the enzyme becomes strongly dependent on the AdoMet concentration (Fig. 3). Threonine synthase (Fig. 1(4)) is half-maximally activated by AdoMet within a similar concentration range (60–210 µM)^{14,15}; this range corresponds to measured pool sizes

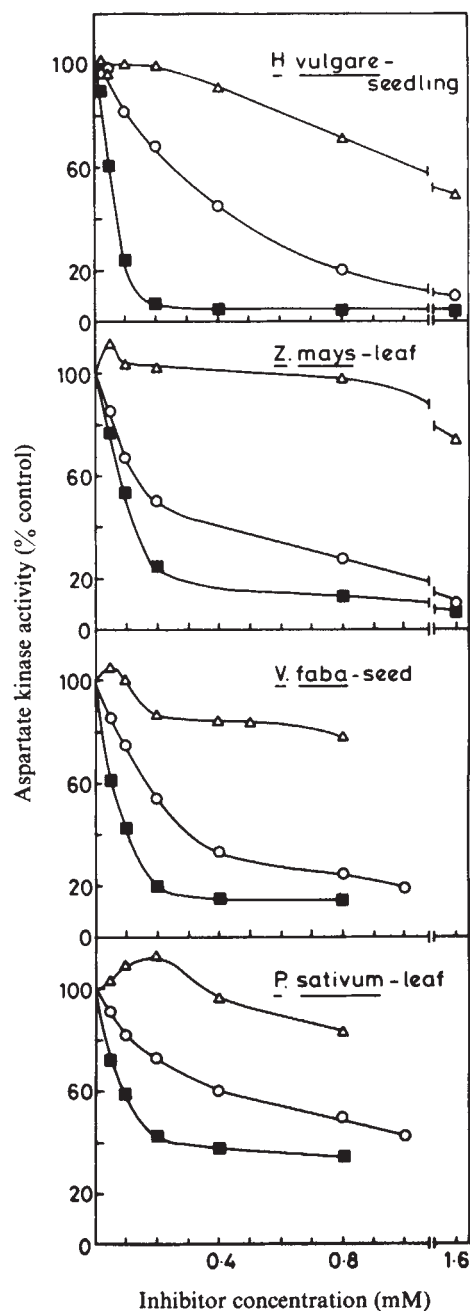


Fig. 2 Synergistic inhibition of higher plant aspartate kinases by Lys and AdoMet. Developing seeds of *Pisum sativum* and *Vicia faba* were collected from pods 18 and 36 days after flowering, respectively. Leaves were collected from light-grown 14-day-old *Zea mays* and 11-day-old *Pisum sativum* seedlings. *Cucumis melo* seedlings were grown in vermiculite for 10 days in light. Excised *Hordeum vulgare* embryos were grown aseptically for 7 days on a sucrose-mineral salts medium. The plant tissues were homogenized and extracted with 50 mM potassium phosphate, pH 7.5, containing 100 mM KCl, 2 mM MgCl₂, 2 mM EDTA, 15% (v/v) glycerol and 0.2% (v/v) 2-mercaptoethanol (buffer A). Supernatant protein extracts after centrifugation (20,000g, 30 min) were fractionated using (NH₄)₂SO₄ (0–60% saturation), gel filtration (Sephadex G-75) and ion exchange (DEAE-cellulose). Aspartate kinase (95–100% Lys-sensitive and Thr-insensitive) from *Hordeum*, *Cucumis* and *Zea* was eluted from DEAE-cellulose using buffer A plus 0.4 M KCl. *Pisum* and *Vicia* extracts were treated as described previously¹². The radioactive aspartate kinase assay⁸ was used. Reaction mixtures contained in 100 µl: 12 mM [U-¹⁴C]aspartate (purified over a Dowex 1-X8 column, 0.7 Ci mol⁻¹), 25 mM MgCl₂, 20 mM ATP, 400 mM NH₄OH-HCl adjusted to pH 7.5 with KOH, 1 mM dithiothreitol, 60–200 µg enzyme protein and various concentrations of Lys and AdoMet (Boehringer Mannheim). Labelled aspartylhydroxamate formed after 60–240 min incubation at 30 °C was determined⁸. Control activities (100%) were (in pkat per mg protein): *Hordeum* 58, *Zea* 28, *Vicia* 40, *Pisum* seed 62, *Pisum* leaf 36, *Cucumis* 48. Δ, AdoMet alone; ○, Lys alone; ■, Lys plus AdoMet (equimolar concentrations).

Table 1 Effect of AdoMet concentration on the sensitivity of plant aspartate kinases to Lys inhibition

[AdoMet] (μM)	Enzyme source					
	<i>P. sativum</i> leaf	<i>P. sativum</i> seed	<i>V. faba</i> seed	<i>Z. mays</i> leaf	<i>H. vulgare</i> seedling	<i>C. melo</i> seedling
	[Lys] required for half-maximal inhibition (μM)					
0	270	240	180	190	340	250
50	130	120	80	ND	80	125
100	70	90	55	95	48	90
250	30	70	20	ND	22	68

Various concentrations of AdoMet and Lys were added to standard assay mixtures. The concentrations of AdoMet used had no significant effect (activity not less than 95% of control) on the enzyme activities when added alone. ND, not determined. For details of assays see Fig. 2 legend.

*in vivo*¹³. Our results suggest that the Lys-sensitive aspartate kinase of higher plants also contains a regulatory binding site(s) for AdoMet. Thus the flux through the pathway may be strongly influenced by AdoMet at Lys concentrations of 20 to 200 μM . A potential consequence of this control is that plants could be starved of Thr unless they contained an isoenzyme of aspartate kinase insensitive to AdoMet and Lys. Although a Lys-insensitive, Thr-sensitive enzyme is found in carrot^{10,11} and legumes^{8,12,18} there is no previous report of such an isoenzyme in cereals. However, our recent but preliminary findings indicate that such an enzyme is present. The likelihood is that, contrary to previous ideas^{4,9}, the control mechanisms for aspartate kinase in higher plants are remarkably conserved between different species, with two isoenzymes being present: one synergistically inhibited by Lys and AdoMet and the other one inhibited by Thr. Understanding the complex regulatory action of the end products is a prerequisite to devising new selection strategies for obtaining mutants of higher plants that produce enhanced levels of amino acids. Further, it is very important in the analysis of mutants already isolated. For instance a mutant of barley has been selected that has a modified Lys, but not AdoMet, site in its aspartate kinase and contains significantly greater amounts of Thr in the seed^{19,20}. The proposed regulation of the aspartate pathway by AdoMet in higher plants differs from that so far reported for microorganisms where Met and/or AdoMet inhibit enzymes specific to the Met branch of the pathway²¹. However, AdoMet may well have regulatory effects, so far unexplored, on other steps of the pathway.

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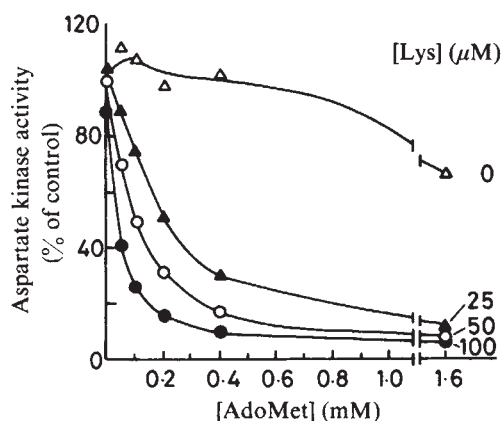


Fig. 3 Effect of Lys concentration on the sensitivity of barley embryo aspartate kinase to AdoMet inhibition. Data obtained as described in Fig. 2 legend.

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DNA from Ti plasmid present in nucleus and absent from plastids of crown gall plant cells

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The Gram-negative soil bacterium *Agrobacterium tumefaciens* induces crown gall tumours on most dicotyledonous plants^{1,2}, its Ti plasmids causing the neoplastic transformation of plant cells^{3–5}. Molecular hybridization has shown that transformed cells contain a segment of the Ti plasmid (T-DNA) of molecular weight 8–15 × 10⁶ (refs 6–9), of which at least part is transcribed^{10,11}. Some copies of the T-DNA are integrated into the plant DNA (unpublished work). In view of the prokaryotic origin of the T-DNA, the possibility of its localization within either mitochondria or chloroplasts must be considered. We present here evidence for the absence of T-DNA from mitochondria and chloroplasts. Our results clearly show its presence within the nucleus.

Two lines of evidence support this conclusion. First, the different organelles were separated and isolated, their DNA was extracted, digested with various restriction endonucleases and hybridized according to the Southern procedure¹² to radioactive probes containing cloned segments of the T-DNA. In the second

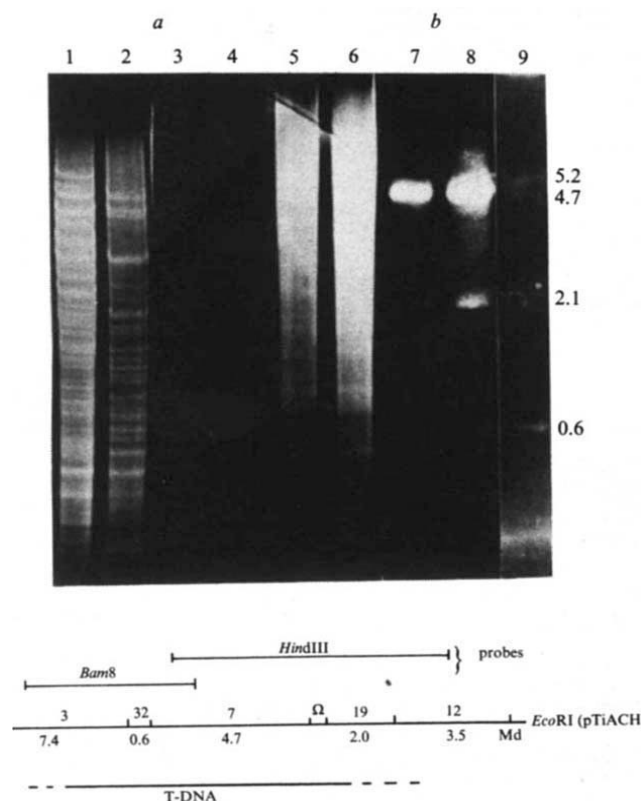


Fig. 1 Hybridization of mitochondrial, chloroplast and nuclear DNA from suspension cultures of *Nicotiana glauca* variety White Burley transformed by *A. tumefaciens* A6 and B6S3 (both lines originated from the collection of R. A. Schilperoort, University of Leiden) against ^{32}P -labelled fragments of pTiACH5. For isolation of mitochondria and chloroplasts, suspension cultured cells were disrupted by a new cell mill (L. Willmitzer *et al.*, in preparation) in buffer A (ref. 13). The homogenates were filtered through an 80- μm and a 20- μm nylon cloth, centrifuged for 10 min at 1,200g, and mitochondria were pelleted by centrifuging the supernatant for 15 min at 12,000g. The pellet was redispersed in buffer A and again centrifuged at 12,000g. The final pellet was treated with DNaseI ($100\text{ }\mu\text{g ml}^{-1}$) for 30 min at 4°C . DNase was removed by two washings at 12,000g in buffer A, and the dispersed pellet was loaded on a 30–60% sucrose gradient in buffer A, centrifuged for 1 h at 22,000 r.p.m. in a SW27 rotor and the mitochondrial band was recovered. Chloroplasts were isolated in the same way but pelleted by two subsequent rounds of centrifugation at 2,000g for 5 min. Nuclei were prepared according to Willmitzer²⁰, substituting 0.5 mM spermidin and 0.15 mM spermin for MgCl_2 , and incubating the cells overnight with cellulase and pectinase. The purity of the nuclei was checked routinely by phase contrast microscopy, which showed the absence of other subcellular particles. The same result was obtained by electron microscopy. DNA was isolated from the organelles according to Gross-Bellard *et al.*²¹ with subsequent purification on a CsCl gradient. DNA was digested with *Eco*RI in 50 mM Tris, pH 7.5, 100 mM NaCl and 7 mM MgCl_2 , and the fragments obtained were separated by electrophoresis in 0.8% agarose formed in 40 mM Tris-acetic acid pH 8.0, 20 mM NaAc, 1 mM EDTA and ethidium bromide ($0.3\text{ }\mu\text{g ml}^{-1}$), on a horizontal slab gel apparatus at 40 V for 12 h²². DNA was then blotted on to nitrocellulose (Schleicher and Schuell) and hybridized against a nick-translated probe of cloned segments of the T-DNA (specific activity $20\text{--}40\text{ }10^6\text{ c.p.m. }\mu\text{g}^{-1}$). Hybridization was performed routinely for 36–40 h at 68°C in $3\times\text{SSC}$, 0.5% SDS, 1 mM EDTA, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin and 0.02% Ficoll. After washing filters were exposed for 1–4 weeks. Lanes 1–4, *Eco*RI restriction pattern of mitochondrial (lane 1) and chloroplast (lane 2) DNA and the corresponding autoradiograms obtained after hybridization with nick-translated *Hind*III fragment 1 of pTiACH5 (lane 3, mitochondrial DNA; lane 4, chloroplast DNA) $15\text{ }\mu\text{g}$ of DNA was applied each. Lanes 5–8, *Eco*RI restriction pattern of two concentrations of nuclear DNA (lane 5, $15\text{ }\mu\text{g}$; lane 6, $22\text{ }\mu\text{g}$) and the corresponding autoradiograms obtained after hybridization with nick-translated *Hind*III fragment 1 of pTiACH5 (lane 7, 8). Lane 9, autoradiogram obtained after hybridization of *Eco*RI digested nuclear DNA with nick-translated *Bam*HI fragment 8 of pTiACH5. The lower intensity of the bands in lane 9 compared with lanes 7 and 8 is due to a lower specific activity of the nick-translated probe. The lower diagram shows a schematic representation of the parts of the Ti plasmid ACH5 (octopine) integrated into nuclear DNA of tobacco cells transformed by *A. tumefaciens* A6 and B6S3. The detailed experiments for the construction of the map will be described elsewhere.

approach, isolated nuclei from transformed cells were incubated in conditions facilitating transcription and the RNA synthesized was analysed for sequences derived from T-DNA.

An efficient method was developed for the isolation of nuclei in plant cell suspension culture, details of which will be described elsewhere (L. Willmitzer, in preparation). Nuclei thus isolated were physiologically active and free from other subcellular particles as revealed by electron microscopy. Standard procedures^{13–15} were followed for the isolation of mitochondria and chloroplasts by DNase treatment and sucrose density centrifugation.

Figure 1a shows the pattern of mitochondrial and chloroplast DNA from a tobacco octopine tumour line transformed by *A. tumefaciens* A6, after digestion with *Eco*RI and separation of the fragments by electrophoresis on 0.8% agarose gels. Figure 1a also shows the corresponding autoradiogram obtained after hybridization with the *Hind*III fragment I of the octopine plasmid pTiACH5, which has been shown to be part of the transferred plasmid DNA (T-DNA)⁹ (De Beuckeleer *et al.*, in preparation). The chloroplast DNA restriction pattern agrees well with reports of the chloroplast pattern of *Nicotiana* species^{16–18}. The mitochondrial DNA shows a higher complexity in comparison with chloroplast DNA as judged by the larger number of distinct bands, in agreement with published data^{15,19}. Mitochondrial DNA preparations were characterized by analytical CsCl density gradient centrifugation, exhibiting a distinct peak at the expected density of 1.706 g cm^{-3} . Lack of hybridization was observed consistently in four independently derived chloroplast and mitochondrial DNA preparations of two different tobacco octopine tumours transformed by *A. tumefaciens* A6 and B6S3.

Figure 1b shows the pattern of nuclear DNA obtained after digestion with *Eco*RI and separation of the resulting fragments by electrophoresis on 0.8% agarose gels, together with the corresponding autoradiogram obtained after hybridization with *Hind*III fragment I and *Bam*HI fragment 8 of pTiACH5. Two bands with molecular weights of 4.7×10^6 and 2.1×10^6 hybridize with the *Hind*III fragment I. These fragments correspond, respectively, to the *Eco*RI fragment 7, molecular weight 4.7×10^6 , of pTiACH5 plasmid DNA which is therefore an internal fragment of the transferred T-DNA, and to a junction fragment presumably linking the right border of the T-DNA to plant DNA (De Beuckeleer *et al.*, in preparation). When *Bam* fragment 8 was used as hybridization probe, three bands with molecular weights of 0.6×10^6 , 4.7×10^6 and 5.2×10^6 emerged, corresponding, respectively, to the internal pTiACH5 *Eco*RI fragments 32 and 7 and the left-hand border fragment (De Beuckeleer *et al.*, in preparation). These data therefore reveal the presence of T-DNA integrated in nuclear DNA.

Hybridization was achieved repeatedly using 11 independently derived preparations of nuclear DNA from two octopine tumour tissues. Because hybridization was achieved with the nuclear DNA, which is enriched by only a factor of 1.1 compared with total cellular DNA, the absence of any hybridization with chloroplast and mitochondrial DNA, which are enriched by a factor of 20 and 100, respectively^{23,24}, suggests that the T-DNA is not located in plasmids. But there is a possible pitfall: because there is extensive homology between 16S and 23S rDNA of *Escherichia coli* and the rDNA of chloroplasts^{25,26} and mitochondria²⁷, erroneous hybridizations were observed when we used cloned T-DNA probes that were not entirely free of chromosomal *E. coli* DNA.

To substantiate further the presence of the T-DNA within the nucleus, isolated and highly purified nuclei from octopine tumour tissue were incubated in conditions that facilitated active transcription. After a 20-min pulse with $\alpha\text{-}^{32}\text{P}$ -UTP, labelled RNA was isolated and hybridized to Southern blots of cloned T-DNA fragments. Figure 2a shows the stained gel together with the corresponding autoradiogram. The T-DNA appears to be transcribed very actively within the isolated nuclei, providing further strong evidence for the presence of the T-DNA within the nucleus. The autoradiogram indicates that the left as well as

the right-hand part of T-DNA are transcribed, for hybridization to the *Hind*III fragments 1, 18 and 22 of pTiACH5 is visible. (The nuclear transcription of the T-DNA is under thorough investigation.)

We excluded the possibility that T-DNA is located in the cytoplasm but adsorbs to and penetrates into the nuclei during isolation. To do this, 20 µg of Ti-plasmid (ACH5) representing a 1,000-fold excess over the concentration present in crown gall cells, assuming five copies of T-DNA per transformed cell²⁸, was added to untransformed plant cells at the onset of nuclear purification. After isolation, nuclei were incubated in conditions facilitating transcription, and ³²P-labelled RNA was isolated and hybridized against blots of T-DNA as described above. No hybridization was detectable (Fig. 2b).

In summary, this report provides strong structural as well as functional evidence for the presence of the T-DNA within the nucleus of the transformed plant cell.

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In vitro suppression of UGA codons in a mitochondrial mRNA

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Although both prokaryotic and eukaryotic messenger RNAs can be easily translated in heterologous protein-synthesizing systems, attempts to achieve correct synthesis of mitochondrial proteins by translation of mitochondrial mRNAs in such systems have failed^{1–3}. In general, the products of synthesis are of low molecular weight and presumably represent fragments of mitochondrial proteins^{1,2}. These fragments display a strong tendency to aggregate⁴. Explanations have included the use by mitochondria of codons requiring a specialized tRNA population⁵ and the fortuitous occurrence within genes of purine-rich sequences resembling bacterial ribosome binding sites⁶. In addition, the long 5'-leader sequences present in many mitochondrial (mt) RNAs may also contribute to difficulties in mRNA recognition by heterologous ribosomes⁷. Recent sequence analysis of human mtDNA⁸ suggests that the genetic code used by mammalian mitochondria deviates in a number of respects from the 'universal' code, the most striking of these being the use of the UGA termination codon to specify tryptophan. That this may also apply in yeast mitochondria has been shown by Fox⁹ and Macino *et al.*¹⁰, thus providing an obvious and easily testable explanation for the inability of heterologous systems to synthesize full-length mitochondrial proteins. We confirm this explanation and describe here the *in vitro* synthesis of a full-length subunit II of yeast cytochrome *c* oxidase in a wheat-germ extract supplemented with a partially purified mitochondrial mRNA for this protein and a UGA-suppressor tRNA from *Schizosaccharomyces pombe*¹¹.

Our choice of mRNA was based on two considerations. First, previous cell-free translation experiments had shown that up to 27% of the products synthesized under the direction of an

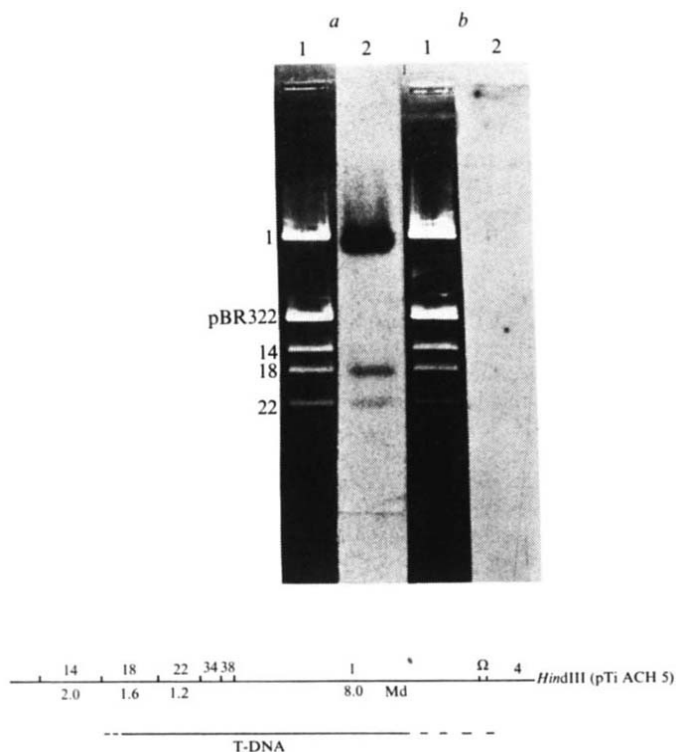


Fig. 2 Transcription of T-DNA in isolated nuclei. Nuclei isolated as described in Fig. 1 were incubated in a buffer containing 15% glycerol, 0.25 M sucrose, 0.1 mM EDTA, 5 mM MgCl₂, 0.5% bovine serum albumin, 60 mM Tris-HCl, pH 8.0, 120 mM (NH₄)₂SO₄ and 0.1 mM GTP, ATP, CTP and 200 µCi α-³²P-UTP (specific activity 350 Ci mmol⁻¹) for 20 min at 25 °C. RNA was isolated by two rounds of DNase digestion, proteinase K treatment, phenolization and subsequent chromatography on Sephadex G50. RNA was hybridized against blots of T-DNA clones containing *Hind*III fragments 1, 14, 18 and 22 at 68 °C in 3 × SSC, 0.2% SDS, 1 mM EDTA for 40 h. **a**, Hybridization (lane 2) of RNA (10 × 10⁶ c.p.m.) transcribed in nuclei isolated from a tobacco crown gall transformed by *A. tumefaciens* A6 to blots of T-DNA clones containing *Hind*III fragments 1, 14, 18 and 22. **b**, Autoradiogram (lane 2) obtained after hybridizing RNA (12 × 10⁶ c.p.m.) transcribed within the nucleus of untransformed cells where 20 µg of Ti-plasmid (pTiACH5) DNA had been added to the cells at the onset of isolation of the nuclei, representing a 1,000-fold excess over the amount of T-DNA present in transformed cells. The lower diagram shows a schematic representation of the parts of the Ti-plasmid ACH5 integrated into nuclear DNA of tobacco cells transformed by *A. tumefaciens* A6.

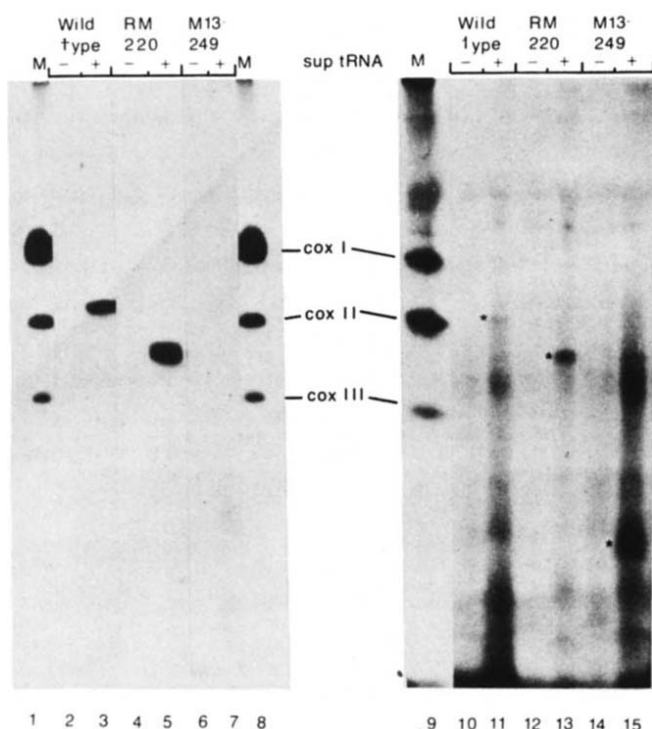


Fig. 1 Autoradiogram of the products synthesized *in vitro* under the direction of mitochondrial mRNA in the presence or absence of tRNA^{sup}_{UGA}. Mitochondrial RNA was isolated from the wild-type strain *Saccharomyces carlsbergensis* NCYC74 and from two mutants in the structural gene for subunit II of cytochrome *c* oxidase, *Saccharomyces cerevisiae* M13-249 and RM220 (refs 9, 12). An 11–13S fraction of these RNAs, which is enriched in the mRNA for subunit II, was obtained by sucrose gradient centrifugation² and used to programme a wheat-germ cell-free system in the presence or absence of tRNA^{sup}_{UGA} from *S. pombe*¹¹. Reaction mixtures (25 μ l) contained 12.5 μ l wheat-germ extract, prepared as described by Marcu *et al.*¹⁶, 20 mM HEPES (pH 7.5), 2.5 mM magnesium acetate, 85 mM potassium acetate, 2 mM dithiothreitol, 0.5 mM spermidine, 0.04 mM of each amino acid, excluding methionine, 3.5 μ M [³⁵S]-methionine (260 Ci mmol⁻¹: The Radiochemical Centre, Amersham, UK), 1 mM ATP, 0.03 mM GTP, 4 mM phosphoenol pyruvate, 4 μ g pyruvate kinase per ml (Boehringer Mannheim), 5 μ M S-adenosylmethionine and 2–3 μ g mitochondrial mRNA. Suppressor tRNA^{sup}_{UGA}, when present, was added to a final concentration of 40 μ g ml⁻¹. Protein synthesis was allowed to proceed for 1 h at 25 °C. 1- μ l samples were withdrawn for measurement of incorporation by trichloroacetic acid precipitation and for electrophoretic analysis of total product. The remainder of the reaction mixture was subjected to immunoprecipitation, following the procedure of Maccacchini *et al.*¹⁷. Immunoprecipitation was carried out in consecutive steps with a pre-immune serum and anti-holo-cytochrome *c* oxidase, using a goat anti-rabbit serum to recover immune complexes in each case. Electrophoresis was performed according to Laemmli¹⁸ on 15% polyacrylamide gels, using subunits I–III of cytochrome *c* oxidase as molecular weight markers. These were prepared by labelling of protoplasts of *S. carlsbergensis* with [³⁵S]-methionine¹⁹ in the presence of cycloheximide (0.6 mg ml⁻¹) after pretreatment with chloramphenicol²⁰. Cell lysates were then immunoprecipitated with anti-cytochrome *c* oxidase as described above. After electrophoresis, gels were fixed, impregnated with sodium salicylate²¹, dried and autoradiographed for 4–8 days at –70 °C on Kodak XR-1 film with an Ilford tungstate intensifier screen. Lanes 2–7, Samples after immunoprecipitation with anti-cytochrome *c* oxidase; lanes 10–15, the total products of *in vitro* synthesis. Lanes 1, 8 and 9 show subunits I–III of cytochrome *c* oxidase, labelled *in vivo* before (lane 9) and after (lanes 1 and 8) immunoprecipitation as described above. The remaining lanes show the products synthesized *in vitro* under the direction of the various mRNA preparations: wild type, in the absence (lanes 2, 10) and presence (lanes 3, 11) of suppressor tRNA; RM220, in the absence (lanes 4, 12) and presence (lanes 5, 13) of suppressor tRNA; M13-249, in the absence (lanes 6, 14) and presence (lanes 7, 15) of suppressor tRNA. Due to the low immune reactivity of the subunit II-related polypeptide in M13-249 (ref. 12), the autoradiogram of lanes 6 and 7 was exposed for a slightly longer time to permit better photographic reproduction. In the electropherograms of total product, the positions of the various forms of subunit II are marked by * cox, Cytochrome *c* oxidase.

11–13S fraction of mtRNA is reactive with an antiserum against cytochrome *c* oxidase^{1,2}. Transcript mapping shows that the 11–13S fraction contains an 11S RNA which is the major transcript of the gene for subunit II on yeast mtDNA (the *OXI-1* locus)². Second, since DNA sequence analysis shows that the *OXI-1* coding sequence is either 753 or 714 nucleotides long⁹, depending on the start used, we reasoned that the 11S RNA with a length of only 800–900 nucleotides would be unlikely to contain extensive untranslated regions. Such an RNA may be more readily accepted by eukaryotic ribosomes than one containing a long 5'-leader characteristic of other mitochondrial mRNAs.

Accordingly, an 11–13S mtRNA fraction was prepared by sucrose gradient centrifugation and translated in a wheat-germ extract as described in Fig. 1 legend. To permit unambiguous identification of the *in vitro* product and to rule out possible artifacts resulting from aggregation, translations were performed not only with RNA from wild-type mitochondria, but also with RNA from two well-defined mutants in *OXI-1*. These mutants synthesize characteristically shortened forms of subunit II as a result of frameshifts induced by single base pair insertions or deletions at known sites in the gene^{9,12}. Figure 1 shows the results obtained from parallel translations in the presence or absence of tRNA^{sup}_{UGA} (~70% pure) from *S. pombe*. Although there is no significant difference in the total amount of trichloroacetic acid-precipitable counts, the presence of the suppressor tRNA results in the appearance of strong, discrete high-molecular-weight bands. These are evident not only in samples precipitated with anti-cytochrome *c* oxidase (lanes 2–7), but also in samples analysed before immunoprecipitation (lanes 10–15). The latter observation indicates that both translation and suppression must be proceeding extremely efficiently and this is quite remarkable in view of the heterologous nature of the mRNA and the fact that, according to the DNA sequence⁹, five UGA stops have to be read through to achieve complete synthesis.

Wild-type subunit II has an *M_r* of 29,000, while RM220 and M13-249 should yield subunit II-related polypeptides with *M_r*'s of 28,000 and 15,000, respectively, as derived from the DNA sequence⁹. In fact, the products synthesized have *M_r*'s of 31,000, 27,000 and 17,000 in wild type, RM220 and M13-249, respectively. We therefore conclude that full-length cytochrome *c* oxidase subunit II has been synthesized and thus are able to confirm that UGA is translated in yeast mitochondria.

The difference in mobility between the product of *in vitro* synthesis with wild-type RNA and the mature *in vivo* subunit II (2,000) deserves comment. The possibility exists that the *in vitro* product is a precursor form of subunit II. Evidence for the existence of a precursor to this subunit has recently been presented¹³. Furthermore, alignment of the yeast amino acid sequence predicted from DNA sequence analysis⁹ with that determined for the beef-heart protein¹⁴, shows that the yeast protein could be larger by up to 27 residues which might represent the non-conserved part of a precursor. However, we cannot formally exclude that this difference merely reflects an altered electrophoretic behaviour arising from the substitution of five tryptophan residues by five serine residues, although this seems unlikely in view of the fact that neither intact subunit II nor the product synthesized in various chain termination mutants shows abnormal behaviour in Ferguson plots of electrophoretic mobility^{12,15}. An alternative explanation is that the extra 2,000 results from initiation in the wheat-germ system at a site upstream of the normal initiation codon. Indeed, DNA sequence analysis has revealed two AUG codons separated by 39 base pairs⁹ and at present it is not known which of these functions as the normal start. Initiation at the 5'-proximal AUG would generate a protein of *M_r* approximately 1,300 greater than one initiated at the second site. These three possibilities can be tested. If the existence of a precursor is confirmed, the present *in vitro* system should be valuable in providing sufficient quantities of this protein to permit further characterization and analysis of its mode of processing.

Finally, the present system has great potential for the identification and characterization of other mRNAs in mitochondria, including those with long 5'-leaders. It will be interesting to see whether their correct translation is achieved. Such experiments are now in progress.

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Cooperative structural transition of PM2 DNA at high ionic strength and its dependence on DNA damages

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Pohl and Jovin¹ have shown that poly(dG-dC)·poly(dG-dC) in solution undergoes a cooperative transition at 2.5 M NaCl at neutral pH. The two forms of the polymer are structurally different as indicated by a change in ethidium bromide intercalation and in circular dichroism (CD)^{1,2}. Wang et al.³ and Davies and Zimmerman⁴ have suggested that the low salt form of the polymer might correspond to the B form of DNA (right-handed helix) and the high salt form to the Z form of DNA (left-handed helix). We describe here a salt-induced transition of supercoiled PM2 DNA from a form which passes through nitrocellulose filters to a form which is retained by these filters. The transition occurs between 2.5 and 3.5 M NaCl. The dependence of the apparent equilibrium constant on the salt concentration indicates a cooperative transition. Irradiation of DNA with UV light or alkylation with *N*-acetoxyacetylaminofluorene (AAAF) shifts the transition to lower salt concentrations. The transition can also be observed with linear DNA but requires a much higher salt concentration.

Figure 1 shows the retention of supercoiled PM2 DNA by nitrocellulose filters in dependence of the NaCl concentration. Relatively little DNA is retained below 2.5 M NaCl. However, at higher salt concentrations, DNA binding rapidly increases and at 3.5 M NaCl 80% binding is obtained. When PM2 DNA is

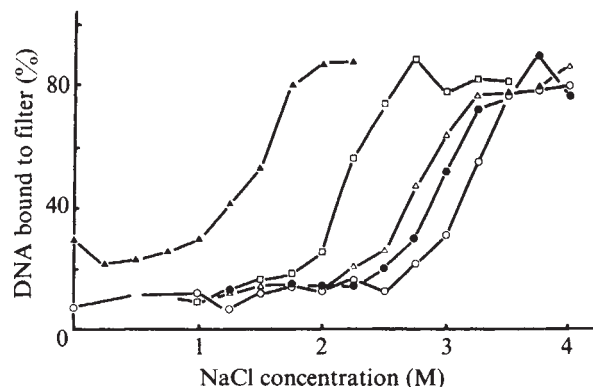


Fig. 1. Retention of supercoiled PM2 DNA by nitrocellulose filters in dependence of the NaCl concentration. ³H-labelled PM2 DNA was isolated as described previously¹⁵. Aliquots (5 ml) of PM2 DNA at concentrations of 0.25 μ M nucleotide in 10 mM Tris-HCl (pH 8.0) and various amounts of NaCl were filtered through nitrocellulose filters (Schleicher and Schuell, 0.20 μ m pore size). The filters were dried and the radioactivity determined. $\circ$, Untreated DNA; $\bullet$, DNA exposed to 60 J m^{-2} of UV light; $\triangle$, DNA exposed to 120 J m^{-2} ; $\square$, DNA exposed to 600 J m^{-2} ; $\blacktriangle$, DNA treated with AAAF. UV treatment was carried out at a concentration of 2.5 μ M DNA nucleotide in a 10 mM Tris-HCl (pH 7.5) on ice. AAAF treatment was at a DNA concentration of 500 μ M with 4.5 $\mu\text{g ml}^{-1}$ of AAAF in 2.3% dimethyl sulphoxide and 10 mM Tris-HCl (pH 7.5). After incubation at 37 $^{\circ}\text{C}$ for 30 min, the DNA was extracted with ether and dialysed against 10 mM Tris-HCl (pH 7.5).

irradiated with UV light or reacted with AAAF, the DNA binding profile shifts to lower salt concentrations (Fig. 1).

DNA binding could be caused by a salt-induced change of the binding properties of the nitrocellulose filters. However, the influence of DNA damages on DNA binding and the observation that linear DNA binds at a much higher salt concentration than circular DNA (Fig. 4) indicate that binding is caused by a salt-induced structural change of the DNA. This structural transition is reversible. When PM2 DNA is exposed to high salt and diluted to low salt concentrations immediately before filtration, no DNA binding is observed. Glass fiber filters (Whatman GF/C) do not retain DNA exposed to high salt, indicating that the DNA is not precipitated. We propose that high salt induces a transition of G-C rich regions of the DNA helix from a B-type structure to a Z-type structure and that the Z-type structure

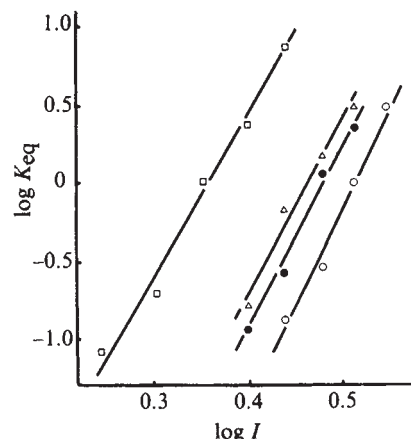


Fig. 2 Double logarithmic plot of the apparent equilibrium constant between the high- and low-salt form of supercoiled PM2 DNA in dependence of the molarity of NaCl (I). The values were calculated from Fig. 1 by dividing the amount of DNA bound to the filter by the amount of DNA passing through the filter. The values were corrected for a fraction of DNA (7.5%) which bound to the filters at all salt concentrations. $\circ$, Untreated DNA; $\bullet$, DNA exposed to 60 J m^{-2} of UV light; $\triangle$, DNA exposed to 120 J m^{-2} of UV light; $\square$, DNA exposed to 600 J m^{-2} of UV light. The slopes varied between 13 and 10 with an average of 11.4.

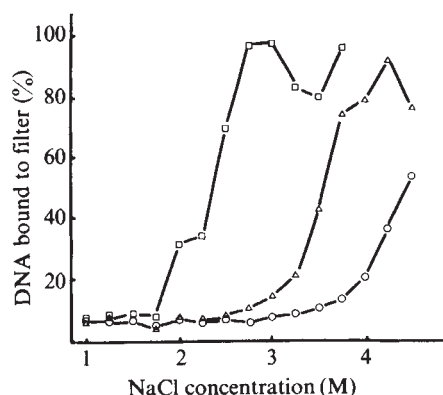


Fig. 3 Dependence of DNA binding on temperature. Aliquots (5 ml) of PM2 DNA at concentrations of $0.25 \mu\text{M}$ nucleotide in 10 mM Tris-HCl (pH 8.0) and various amounts of salt were incubated at 50°C ($\square$), 23°C ($\triangle$) and 0°C ($\circ$). They were then filtered through nitrocellulose filters which had been prewashed with 5 ml salt solutions at the same temperatures. The filter speed was 9 s per 5 ml.

itself, or single-strand regions formed in junctions between B and Z segments, bind the DNA to the nitrocellulose filter.

A double logarithmic plot of the apparent equilibrium constant (DNA bound to the filter divided by DNA passing through the filter), according to the equation $\log K = r \times \log(I) + \text{constant}$, where I = molar NaCl, gives approximately straight lines with slopes between 13 and 10 (Fig. 2). The high value of r indicates that the transition is cooperative. The average value of 11 is the same as that reported by Pohl and Jovin¹ for the salt-induced cooperative transition of poly(dG-dC)·poly(dG-dC) with an average length of 34 base pairs.

CD studies indicate that Z DNA segments can also be induced by high salt in naturally occurring DNA^{5,6}. Changes of the CD spectrum similar to those induced by high salt have also been observed in DNA or in poly(dG-dC)·poly(dG-dC) alkylated with mitomycin C⁷. This observation and our observation with AAF- and UV-treated DNA suggest that DNA damages facilitate the transition of the DNA helix from a B form to a Z form.

The Z form in damaged DNA might be favoured for steric reasons. Thus, in oligonucleotides *N*-(deoxyguanosin-8-yl)-acetylaminofluorene, the major adduct in AAF-treated DNA, assumes the *syn* configuration found in the Z DNA rather than the *trans* configuration found in B DNA⁸. Alternatively, a DNA

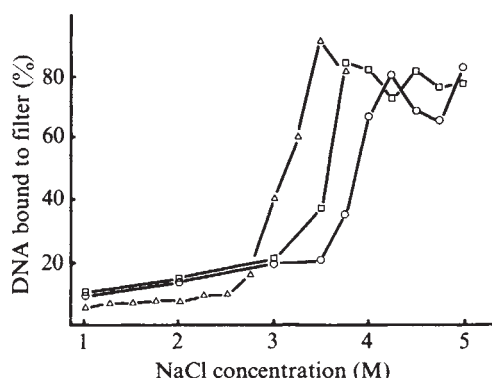


Fig. 4 Retention of native and linear PM2 DNA at 37°C in dependence of the NaCl concentration. $\triangle$, Native PM2 DNA; $\circ$, linear PM2 DNA; $\square$, linear PM2 DNA exposed to 600 J m^{-2} of UV light. Linear DNA was prepared by incubating 0.2 mM PM2 DNA nucleotide for 3.5 h with 25 units ml^{-1} of restriction endonuclease *MspI* (New England Biolabs) in 10 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 6 mM KCl and $100 \mu\text{g ml}^{-1}$ acetylated bovine serum albumin. After incubation, the DNA was extracted with an equal volume of chloroform-octanol (9:1) and dialysed against 10 mM Tris-HCl (pH 7.5). Filtrations were carried out as described in Fig. 3 legend.

structure containing B and Z segments might require that the hydrogen bonding between some base pairs is disrupted. DNA damages might therefore favour the Z structure because of interference with hydrogen bonding. Indeed, AAF treatment and UV treatment lower the melting point of DNA^{9,10}. Furthermore, an increase in temperature leads to a transition at a lower salt concentration (Fig. 3). The influence of temperature on the transition is not at the level of the formation of Z form itself (at least in regions with an alternating G-C sequence), as the transition in poly(dG-dC)·poly(dG-dC) is not temperature-dependent¹.

The transition of segments of the DNA from a B form to a Z form requires unwinding of the DNA helix. In a negatively supercoiled DNA molecule, unwinding of the DNA will remove the supercoils and, depending on the degree of transition, introduce positive supercoils. Since removal of negative supercoils is associated with a considerable decrease in free energy^{11,12}, one would expect that the B-Z transition is favoured in DNA containing negative supercoils. As shown in Fig. 4, when the negatively supercoiled PM2 DNA is converted to a linear form by treatment with the restriction endonuclease *MspI* (*MspI* is an isoschizomer of *HpaII* which cleaves PM2 DNA at a unique site^{13,14}), the transition is induced at a much higher salt concentration.

It will be of interest to demonstrate that Z DNA-like structures also occur at physiological salt concentrations. Indeed, this is the case for DNA damaged extensively with AAF or with mitomycin C, and, with the appropriate DNA binding proteins present, the transition might also occur with DNA containing fewer lesions. Such Z DNA-like structures might be the trigger for the initiation of DNA repair processes. One would predict from such a mechanism that the presence of negative supercoils is important for DNA repair—it has been demonstrated in *Escherichia coli* that gyrase antagonists which prevent negative supercoiling inhibit the restoration of infectivity of UV-irradiated phage λ DNA¹⁵. Transitions of DNA segments from a B form to a Z form and vice versa might also be involved in other aspects of regulation of DNA metabolism, a mechanism which would be compatible with the key role of DNA gyrase in *E. coli*¹⁶.

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Corrigendum

In the letter ' β -Endorphin alters luteinizing hormone secretion via the amygdala but not the hypothalamus' by N. Parvizi and F. Ellendorff, *Nature* **286**, 812–813 in paragraph 2, lines 2/3, the reference to 'Basolateral hypothalamus' is incorrect and it should read 'mediobasal hypothalamus'.

MATTERS ARISING

Giant solar flares in Antarctic ice

ROOD *et al.*¹ have discovered four prominent 'spikes' in a long time record (circa 1150 to the present) of the NO_3^- concentration inside an Antarctic ice core. These four spikes rise 2–3 times higher than the upper envelope of a fluctuating background level of $0\text{--}20\ \mu\text{g l}^{-1}$ that has been plausibly attributed to the action of high-energy solar radiation (photons and particles) impinging on the Earth's upper atmosphere and ionizing N_2 , thereby leading to various chains of chemical reactions that culminate in the formation of NO_3^- , some of which is transported, within a few weeks or months, to Antarctica¹. According to three alternative chronologies provided by Rood *et al.*, the estimated dates of the four NO_3^- spikes lie within the intervals given in Table 1. Three of these dates have been tentatively associated by the same authors with the galactic supernovae of 1604, 1572 and 1181. At the outset, they have rejected energetic particles from the supernova explosion as a possible source of ionization of the terrestrial N_2 because galactic magnetic fields would have greatly delayed and diffused the particles on their way to Earth. Instead, they have shown that photons of energy $\geq 10\text{ keV}$ are required. Unfortunately, as they admitted, the total energy requirements are difficult to meet, and the matching of dates with historical supernovae is not perfect.

As an alternative explanation, I suggest that the necessary ionizing radiation could have come from unusually powerful solar flares. These flares would be expected to have occurred preferentially during periods when the Sun was generally most active, that is around the times of the largest maxima in the solar cycle. Two good indices of solar activity are available: for the years elapsed since 1700, there are both sunspot numbers² and auroral numbers³; for earlier years auroral statistics^{3–6} are preferred because the sunspot record (mostly from the Far East) is very sporadic⁷. Despite some incompleteness of the record before 1700, the main trends in the statistics are quite unmistakable (see refs 3 and 5).

The intervals of time in which the largest auroral and sunspot maxima occurred are listed in Table 1, where earlier dates are given only to the nearest half-decade. These intervals of time correlate very well with the known epochs of the NO_3^- spikes. Only in one case is it necessary to recognize that episodes of solar flaring need not occur (as they have not always occurred in modern times) precisely at times of maximum auroral or

maximum sunspot numbers. This therefore gives some flexibility in the possible dates of the giant solar flares that is not available in the case of the supernova hypothesis. However, there are no NO_3^- spikes in the years around 1778 and 1957, at which times solar activity was also at a peak. Nevertheless, given the rarity of the proposed flaring events, this absence could simply be a product of statistical fluctuations. Also the Sun may now be somewhat different physically from its state before the long Maunder minimum in 1645–1715 (ref. 2).

According to Table 1, very large solar maxima seem to recur in cycles of ~ 200 yr, as Schöve⁴ originally noted. In the background NO_3^- data, there is also some evidence of the Maunder minimum and of the normal 11-yr solar cycle¹. Bauer⁸ estimated that the background concentration could vary by a factor of two during the 11-yr cycle. To extend this further, I consider the largest solar flares in modern times. These have importance class 3+ or 4 and emit $E_{32} \times 10^{32}$ erg of high-energy radiation (photons and particles), where $E_{32} \sim 1\text{--}2$ (ref. 9). The Earth intercepts $4 \times 10^4 E_{32}$ erg cm^{-2} of this. According to Rood *et al.*¹, the energy flux needed to produce a NO_3^- concentration of $20\ C_{20}\ \mu\text{g l}^{-1}$ near the South Pole is $\sim 1 \times 10^5 C_{20}$ erg $\text{cm}^{-2}\text{ yr}^{-1}$. Since $C_{20} \leq 1$, only one or two major flares per year is sufficient to produce all of the background NO_3^- . It is therefore not unreasonable to suppose that, every couple of hundred years or so, a giant flare, perhaps 2–3 times more intense than ordinary major flares, erupts on the Sun. An alternative possibility is that a very rapid succession of major flares of the ordinary type takes place. An event of this type may still develop during the current maximum of solar activity.

The Antarctic ice-core measurements are apparently now being pushed to deeper levels than before¹. Much older NO_3^- spikes may therefore be discovered. One immediate prediction of the solar flare hypothesis is the possibility (though no more than that) of a spike occurring around the year 1000, a time of height-

ened auroral activity^{3–5}. On the other hand, a very bright supernova also appeared in 1006, as Rood *et al.* pointed out. But, fortunately, an *experimentum crucis* to discriminate between the two hypotheses can be made for the middle of the eleventh century, a time of profound auroral quiet but, equally importantly, of a brilliant supernova, the Crab explosion of 1054.

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Magnetic fields and the solar constant

THOMAS¹ has suggested that changes in the magnetic flux content of the convection zone produces changes in radius. However, his calculations did not include the effect of structural changes in the superadiabatic region on the bulk of the convection zone. Theoretical studies^{2–4} have shown that solar luminosity fluctuations can result from small structure adjustments in the convection zone, and can occur on time scales shorter than 1 yr. Such fluctuations are of interest in studies of the terrestrial climate.

The previous calculations have forced these structural changes by assuming a time-dependent mixing length. When the mixing length (physically the convective efficiency) changes, adjustments in the structure occur rapidly in the superadiabatic region, and in turn the bulk of the convection zone adjusts to maintain hydrostatic equilibrium. The result is a temporary luminosity change³. Any mechanism which affects the structure of the superadiabatic region will cause such a luminosity fluctuation. The effect of magnetic pressure changes on the superadiabatic region is described here.

We began by including a global magnetic pressure term in a stellar structure code. If the flux is assumed to be concentrated in vertical flux tubes, the pressure at a given radius (r) is given by

Table 1 Dates of the largest maxima in the Antarctic concentration of NO_3^- and in solar activity indices

Largest NO_3^- maxima	Largest solar maxima
1130–1160	1120–1140
1300–1340	1360–1375
1590–1600	1565–1585
1610–1620	1605–1630
–	1778–1788
–	1947–1959

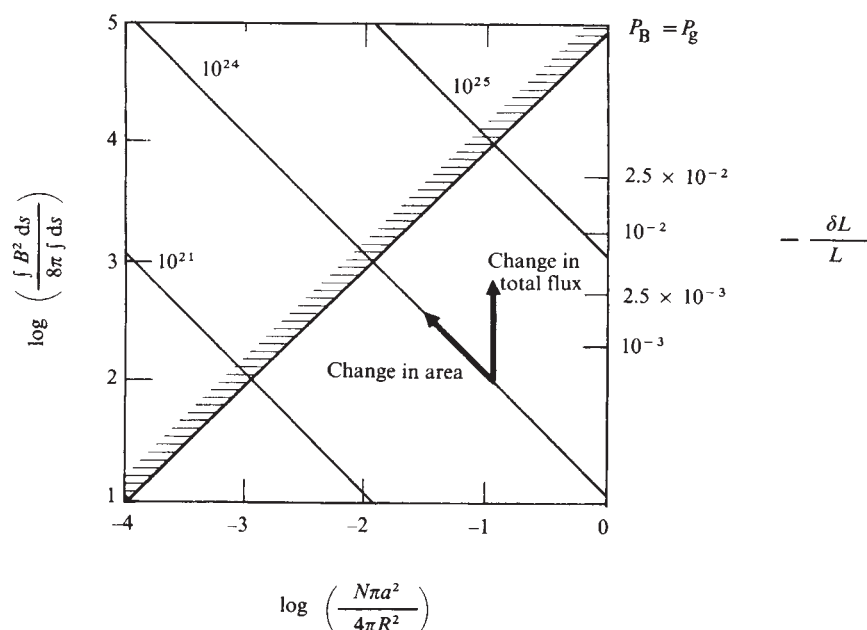


Fig. 1 Global magnetic pressure plotted against the fraction of the solar surface contained in the magnetic flux tubes. The luminosity scale on the right side gives the change resulting from the magnetic pressure change. The oblique lines are contours of constant flux.

$$P_m = \frac{1/8\pi \int B^2 ds}{\int ds} \approx \frac{NB^2 a^2}{32\pi r^2}$$

where N is the number of tubes with flux B and radius a . If the flux in a tube is constant and material is locked to the field lines, then the ratio of magnetic pressure to gas pressure decreases rapidly with depth in agreement with detailed sunspot models by Weiss⁶.

The effect of changing the value of the magnetic pressure was explored by changing the surface value through the range $\log P_m = 1$ to 3.8 dyn cm^{-2} . Only in the superadiabatic region (upper few thousand kilometres) was the magnetic pressure term significant. Its gradient results in expansion and decreased density. In response to the lowered density in the superadiabatic region, the bulk of the convection zone begins to expand. The energy for expansion is obtained from the luminosity, and thus increasing the magnetic pressure decreases the solar luminosity.

The magnetic pressure depends on the total magnetic flux present and the area containing that flux. However, there is a minimum area over which a given amount of flux may be distributed that is determined by the requirement that the magnetic pressure in the flux tubes should not be greater than the gas pressure outside the tube.

The surface value of the global magnetic pressure is plotted against the fraction of the solar surface contained in the magnetic flux tubes in Fig. 1. Lines of constant total flux are shown as diagonals and the effect on the pressure of changing the area contained in flux tubes can be seen by moving along one of these lines. The effect on the pressure of changing the

total amount of flux contained in the flux tubes is shown by vertical motion in this diagram. The luminosity scale along the right side of Fig. 1 gives the amplitude of the luminosity fluctuation produced by the magnetic pressure change.

The solar budget of magnetic flux is uncertain, but to produce a luminosity change of the order of 0.1% the flux penetrating the superadiabatic region must be $\geq 2 \times 10^{23} \text{ Mx}$. The radius change associated with this flux change was found to be $\Delta R/R \leq 10^{-4}$ which is slightly less than the value found by Thomas¹ for two reasons. First, the vertical flux tubes considered here contain less volume through the superadiabatic region than the horizontal flux tubes considered by Thomas. Second, the radius of the upper parts of the superadiabatic region is affected by the luminosity change because energy transport through the outer 1,000 km is partially by radiation^{3,4}. Thus reducing the radiative flux causes this region to contract.

While the total amount of magnetic flux in the convection zone is uncertain, the amount of flux required for its pressure to produce a significant luminosity change is apparently high. A 1% luminosity change required a flux change of $2 \times 10^{24} \text{ Mx}$ in our model, and resulted in a radius change of $\Delta R/R = 2 \times 10^{-4}$. The action of such large amounts of magnetic flux on convective energy transport may actually dominate the effects discussed here. Unfortunately, although it is well known that strong fields can suppress convection locally (sunspots), the realistic inclusion of the effects of such flux tubes on global convective energy transport is a problem. As the effects of the field on convection have been neglected, the present results should represent the minimum effect of

magnetic fields on the solar luminosity and radius.

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A tectonic mélange of foreign eclogites and ultramafites in West Norway

MEDARIS¹ suggests that eclogites enclosed within the amphibolite-facies country-rock gneisses in the Basal Gneiss Region, West Norway "developed by *in situ* metamorphism of crustal materials"¹; furthermore he attributes this to an "eclogite-facies metamorphic event"¹. There are, however, some weaknesses in these hypotheses; also the simple *in situ* hypothesis may detract other geologists, especially geodynamicists, from pondering the genesis of these rocks, some of which are amongst the most exceptional on Earth², being the highest-pressure metamorphic rocks in the Caledonide Orogen³.

Many different kinds of eclogite can be distinguished in the region according to mineralogical, textural, chemical, and pressure (P)-temperature (T) criteria, such that the frequent assumption^{1,4-8} of a single origin (the simple *in situ* origin) for all eclogites within country-rock gneiss is difficult to justify. The 'dual' origin of eclogites and ultramafites, with only the ultramafites being foreign, suggested at Lien¹ and elsewhere^{7,9}, is less credible than my alternative multiple foreign origin¹⁰ of the different eclogite types and also of those meta-igneous rocks (ultramafites, anorthosites, dolerites) for which adequate evidence of igneous intrusion into country-rock gneiss is unavailable (most published examples). A gigantic tectonic mélange is thus envisaged having been created by tectonic introduction of rock fragments from diverse foreign sources of diverse ages of equilibration [for example eclogite-facies eclogites + ultramafites (upper mantle); granulite-facies eclogites + anorthosites + gneisses + autometamorphosed dolerites

(lower continental crust); blueschist-facies eclogites (subducting ocean crust); Ulstein-Dimnøy eclogite (subducted ocean crust); Ulstein-Dimnøy "garnet-biotite-gneiss" (subducted argillaceous sediments); marbles (subducted carbonate sediments)] into preserved Precambrian amphibolite-facies country-rock gneisses (also occasionally into each other) by lithospheric interdigitation during two-continent collision^{11,12}. The final emplacement event was presumably of Caledonide age^{12,13}. Age relationships between the diverse rock types in the Basal Gneiss Region are noted for their complexity^{8,12}; the tectonic events deduced here would have produced just such a complexity.

Concerning the Lien locality, of the three rock types described by Medaris¹ (an ultramafite boudin, eclogite boudins, and country-rock gneisses which envelop the boudins), P - T estimates are provided¹ only for the boudins. No evidence is presented for P - T estimates for the country-rock gneisses, although demonstration of the identicalness of P - T and time conditions for equilibration of the eclogites and country-rock gneisses is essential^{11,12} to sustain the deduced¹ *in situ* model. The use¹ of assumed co-existing core mineral compositions for geothermobarometry must be suspect when the cores are, by definition, not co-existing, especially when the raw data on zoning patterns is not provided for critical assessment of possible coexistence; confident interpretation of mineral zoning is notoriously difficult, for example in garnet¹⁴.

Low- P estimates^{1,6,15} for eclogite equilibration derive mainly from the intersection of iso- $K_D^{Fe/Mg}$ P - T dependent lines with iso- X_{cp}^{Jd} minimum P -variable T lines yielding minimum P only. Medaris¹ follows Krogh^{6,7} in regarding only these minimum P estimates (7–12 kbar) as reflecting the true P . Their trends are thus only consistent¹ in their method of derivation, which would yield a linear positive dP/dT trend for any set of eclogites with variable $K_D^{Fe/Mg}$ and approximately constant X_{cp}^{Jd} . Their published data^{1,6} are perfectly compatible with any P estimates along the iso- K_D lines, easily 10–20 kbar higher than the minima (compare with 15–40 kbar, estimated for other eclogite boudins from the same district¹²). A near-infinite variety of P - T trends is thus possible, including depressurization trends; the supposed "prograde" trend, said¹ to have been "established by Krogh⁶", clearly needs independent corroboration. Medaris¹ claims convergence of his eclogite and ultramafite P - T trends, but they barely converge in his Fig. 2. His inferred eclogite trend¹ lies alongside the basic granulite/eclogite transition⁴ based on the low- T linear extrapolation of high- T experimental work¹⁶, an extrapolation inconsistent with other data^{17–19} which suggests

higher P at low T . Even if the documented¹ prograde eclogite P - T trend at Lien¹, or regionally^{6–8}, is genuine, it contributes little to support the *in situ* hypothesis if an identical trend is not discernible in the country-rock gneisses, evidence for which is apparently completely lacking. Whilst recognizing the feasibility of generating eclogite parageneses and granulite- or amphibolite-facies gneisses *in situ* from adjacent basaltic and granitic rocks in certain P - T -composition conditions, I would anticipate the common occurrence of co-existing omphacite + plagioclase in intermediate compositions, but I am not aware of their existence in the country-rock gneisses of this region.

High- P equilibration (> 15 kbar) of at least some eclogite boudins has been implied or specifically estimated by advocates of the foreign tectonic emplacement origin^{2,3, 11,12, 17, 20–22, 24,25}, whilst low P equilibration (< 15 kbar) for all eclogite boudins has been implied or specifically estimated for some by advocates of the *in situ* origin^{1, 4,5, 9, 15, 26–28}. Krogh⁶ broke through the < 15 kbar threshold by proposing P estimates up to 23 kbar. The significant fact that this estimate⁶ lies beyond¹¹ the known stability field of any plagioclase composition has been bypassed⁷ and apparently disregarded¹. Hence accepting^{1,7,8} Krogh's⁶ postulated prograde regional P - T trend necessitates the annihilation, kinetics permitting, of each grain of abundant plagioclase in $\approx 4,000 \text{ km}^2$ of country-rock gneisses, yielding ubiquitous eclogite-facies parageneses. The expected evidence for this substantial event is notable for its paucity and dubious validity, apparently being confined^{7,8} to depressurization symplectites after omphacite in gneisses around the Ulstein-Dimnøy eclogite^{5,15}. Considering published data only, and noting especially: (1) the outcrop of the symplectite-bearing 'garnet-biotite-gneiss'^{5,15,29} which occurs only in contact with this eclogite^{15,29}, and which is "remarkably garnet-rich"⁵; (2) the lack of symplectites reported specifically from the 'dioritic'^{5,15,29} typical country-rock gneisses, which are more deformed²⁹ and migmatitic⁵, and (3) the 'uncommon'⁵ amphibolitized margins of the eclogite boudin, and its 'welded'¹⁵ contacts, allows, and indeed supports, my alternative interpretation that the so-called 'garnet-biotite-gneiss' is not real country-rock gneiss, but is merely a contiguous layer of amphibolitized K-, Al- and Si-rich quartz-phengite-eclogite tectonically independent of the 'dioritic' real country-rock gneiss. If this is true, then the assertion¹⁵ that this eclogite is a type example of *in situ* metamorphism would be unsubstantiated. Furthermore, taking the absence of plagioclase as one of the essential features of an eclogite-facies group of parageneses², the suggested "eclogite-facies metamorphic event"¹¹ within the country-rock gneisses would be as

unfounded as the postulated depression of the country-rock gneisses to depths of $\approx 70 \text{ km}$ ^{1,6,8}.

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MEDARIS REPLIES—There is increasing evidence in the Basal Gneiss terrane of western Norway for high-pressure metamorphism due to tectonic thickening of continental crust, probably related to a continent-continent collision of Caledonian age^{1–3}. Thus, Smith's suggestion that the Basal Gneiss terrane has originated as a gigantic tectonic mélange due to lithospheric interdigitation during a continent-continent collision deserves evaluation by further field and petrological investigations. Such a model provides a plausible mechanism for emplacement of large ultramafic bodies, such as that at Lien, and would be consistent with the P - T patterns preserved in Norwegian garnet peridotites^{4–6}.

As Smith has suggested, there may be diverse origins and histories for country-rock eclogites in the Basal Gneiss. Nevertheless, there is now compelling chemical, petrological, textural, and structural evidence^{1–3} for *in situ* prograde metamorphism of at least some of the eclogites, and the country-rock eclogites at Lien properly belong in this group.

Unfortunately, I feel that Smith has misrepresented three points in my paper. (1) The garnet-bearing ultramafic rocks and country-rock eclogites are relicts of an earlier eclogite metamorphism that are now surrounded by chlorite peridotite and

middle grade gneisses, respectively, and the eclogite-facies rocks themselves have been retrograded to assemblages that are compatible with those in the enclosing rocks. (2) Admittedly, there is no certainty that equilibrium has been preserved between mineral cores. However, mineral cores have relatively uniform compositions within each sample, and different mineral grains within each sample yield consistent core temperatures⁶. (3) I clearly stated that pressure estimates for the country-rock eclogites at Lien, based on the jadeite content of omphacite, are minimum values only. Although many of Krogh's pressure estimates for eclogites in the Basal Gneiss terrane are minimum values, as he stipulated, others are true estimates, based on the coexistence of orthopyroxene or phengite with garnet and omphacite.

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GABA mediated circling from substantia nigra

THE dialogue between Waddington¹ and Martin and Haubrich² emphasizes the complexity of the interaction between striatal dopamine systems and nigral γ -aminobutyric acid (GABA) mechanisms as recently pointed out by Arnt and Scheel-Kruger³. This is particularly true when interpreting behavioural changes in terms of biochemical parameters. The effects of manipulating nigral GABA mechanisms on striatal dopamine release are complex. While nigral application of GABAergic drugs, such as muscimol, increases the firing rate of dopaminergic neurones⁴, and the release of dopamine in the ipsilateral caudate nucleus^{5–7}, GABA itself exerts a biphasic action first stimulating and then inhibiting striatal dopamine release⁸. Furthermore, the nigral application of picrotoxin stimulates dopamine release in the striatum, an effect which is prevented or reversed by diazepam⁹, which itself decreases dopamine release in striatum¹⁰ (presumably by facilitation of nigral GABA transmission). Some GABAergic terminals (possibly those projecting to zona compacta) therefore seem to inhibit the activity of nigral dopamine cells, while others (possibly those projecting to zona reticulata) may indirectly activate the same nigral dopamine cells, perhaps via a

GABA interneurone. Whether turning behaviour induced by the intranigral administration of muscimol is due indirectly to alteration of dopamine release in the striatum is disputed. In fact, the effect of intranigral injection of GABAergic compounds is critically dependent on the exact site of injection, an issue which has been neglected in the correspondence on this topic.

The initial observation of Tarsy and his colleagues¹¹ that picrotoxin injected into substantia nigra zona compacta caused contraversive rotation was followed by the discovery of Scheel-Kruger *et al.*¹² that ipsiversive circling resulted from the injection of picrotoxin into a caudal nigral site. Subsequently, James and Starr¹³ (and, independently, ourselves¹⁴) demonstrated that the direction of rotation produced by injection of picrotoxin into rostral nigra is reversed on injection of this compound into caudal nigra, suggesting the existence of separate GABA mechanisms. We obtained similar findings injecting picrotoxin, and also muscimol in caudal and rostral nigra.

The circling produced by the action of muscimol or picrotoxin at each nigral site can be distinguished on the basis of its dependence on intact striatal dopamine mechanisms. Thus, while circling induced by injection of GABA-active compounds into rostral nigra is reduced by 6-hydroxydopamine lesions of the nigro-striatal pathway, or by systemically administered haloperidol, the same treatments are ineffective in inhibiting circling induced by manipulation of GABA mechanisms in caudal nigra^{1,13,14}. These findings support Waddington's assertions of non-dopamine-mediated contraversive circling being induced by muscimol injected into substantia nigra zona reticulata¹. The exact site used by Martin and Haubrich⁵ is not clear, but must have involved caudal nigra as muscimol induced contraversive rotation. However, the muscimol perfused into substantia nigra also may have reached GABA receptors in rostral nigra to activate the ascending nigro-striatal pathway so causing increased striatal dopamine release. This would account for the simultaneous occurrence of these phenomena.

The argument as to whether or not the striatodopamine release caused by intranigral muscimol is responsible for contraversive circling is redundant. The circling behaviour initiated by an asymmetry of striatal dopamine function is clearly mediated by strio-nigral fibres and the substantia nigra. Thus, lesioning of the descending strio-nigral pathway prevents contraversive circling behaviour induced by the administration of apomorphine to animals with a unilateral 6-hydroxydopamine lesion of the nigro-striatal pathway¹⁵. In addition, kainic acid-induced destruction of neurones in substantia nigra zona reticulata, or the

injection of picrotoxin or bicuculline into nigra ipsilateral to a 6-hydroxydopamine lesion of the nigro-striatal dopamine pathway, results in reduction, abolition or reversal of the contraversive rotation produced by apomorphine¹⁶. All these data point to a primary role of a neuronal pathway from caudal nigra independent of dopamine, which is responsible for the mediation of the circling phenomena. The importance of this nigral mechanism is emphasized by the work of Papadopoulos and Huston¹⁷ who showed that spontaneous circling behaviour induced by a unilateral electrolytic lesion of substantia nigra was not influenced by the removal of the telencephalon (including striatum and neocortex). Indeed, we have demonstrated that a hemi-transection rostral to nigra severing all rostral nigral afferents and efferents does not attenuate contraversive circling induced by intranigral administration of muscimol (unpublished observations).

In conclusion, we support Waddington in his assertion that the contraversive circling due to intranigral muscimol administration is not due to striatal events. There can be little doubt as to the importance of a non-dopaminergic nigral output pathway in the mediation of circling behaviour. The nature of this pathway remains unknown as does its target site. It does not seem to involve the known projections of the nigra to the superior colliculus¹⁸ or thalamus¹⁹ and we are now investigating the involvement of nigro-reticular pathways.

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BOOK REVIEWS

A question of language

Jean Berko Gleason

WHY would anyone want to try to teach a chimpanzee the sign language used by deaf human beings? For at least two compelling reasons. The first has to do with linguistic questions: is language a strictly human phenomenon, or is it possible that intelligent animals such as chimps, which are physiologically unable to speak, are nonetheless capable of combining symbols in a structured, grammatical way? The second reason is related to what Donald R. Griffin calls "the question of animal awareness": is it possible that we can teach non-human creatures to communicate to us their ideas, thoughts, their world view?

These tantalizing questions about language and cognition are what motivated Herbert S. Terrace, a Professor of Psychology at Columbia University, to adopt an infant male chimpanzee, whom he named Nim Chimpsky (Nim for short) after the linguist Noam Chomsky, a proponent of the view that only humans possess the special innate abilities that make true language possible. According to this view, language is a species-specific capacity, and all human languages share certain grammatical similarities because of the constraints imposed by the human intellect. Thus, all languages are

Nim: A Chimpanzee Who Learned Sign Language. By Herbert S. Terrace. Pp. 384. (Knopf: New York/Eyre Methuen: London, 1980.) \$15, £6.50.

composed of a small number of elements that can be combined in a great variety of utterances, have classes of words that roughly correspond to nouns and verbs, and so on. The fact that only humans possess language and that human infants master the complexities of their native tongue in just three or four years in a fairly effortless way and without extensive tutelage is evidence for the innatist view. By contrast, behaviourists hold that language is not basically different from other behaviours, and that its presence depends primarily on establishing a suitable system of rewards for linguistic behaviour.

Terrace himself has an extensive background in the behaviourist tradition, and in preparation for this project familiarized himself with the literature on language acquisition by children. His plan was to raise Nim in a rewarding human environment, teaching him American Sign Language (ASL), and then to analyse in a rigorous and linguistic way the young

chimp's emerging ability to produce one sign at a time and later to combine signs into longer 'utterances'. There are, of course, other apes who have learned sign language, the first and most famous being Beatrice and Allen Gardner's Washoe; and there are apes who have learned to produce certain kinds of messages by manipulating plastic symbols or by striking the appropriate keys on a computer console. Project Nim was to go beyond these by providing a detailed record of Nim's acquisition of ASL, paying special attention to any evidence that he could produce grammatical signed sentences.

While the book gives a good picture of what it is like to live with an ape in New York City, and details the problems the project had in recruiting and keeping the 60 dedicated ASL teachers who worked with Nim, the essence of the book lies in its linguistic argument. The chimp's signed utterances were analysed for evidence of structure in much the same way as is young children's spoken language. By comparison, the chimp was found wanting. Even though he amazed everyone by producing his first ASL sign (*drink*) before the age of four months, his later utterances never went much beyond the two-sign stage. Reviewing the recorded utterances, the author concludes that "his three-sign combinations do not . . . provide new information. Consider, for example, Nim's most frequent two- and three-sign combinations: *play me* and *play me Nim*. Adding *Nim* to *play me* is simply redundant". Since tables of the most frequent combinations are provided, it is possible for the reader to do some armchair analysis, and in this case the conclusion appears to be based on a rather selective view of the data; there are a number of instances listed where a three-sign combination seems to build on earlier two-sign combinations in a way that very much resembles children's language. At the two-sign stage, for instance, Nim said *eat Nim*, while the three-sign list contains elaborations like *more eat Nim* and *banana eat Nim*. This begins to look like structure, but the four-sign combinations are not only very rare, they are all of the redundant *eat drink eat drink* variety. Most of Nim's signing had to do with eating, drinking and playing, but from time to time we are given a glimpse of the young chimp paging through a book and softly signing to himself the names of the things he sees.



Nim at work with one of his teachers

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In the end, the author concludes that there is no evidence that chimps can produce sentences. Moreover, analysis of the project's videotapes indicated that most of his signed utterances were prompted by a teacher and contained major constituents of the teacher's previous utterance. Only 10% of Nim's utterances were spontaneous. Furthermore, he showed little ability to hold a conversation; he tended to interrupt his teachers frequently and to be unaware of the notion of taking turns.

Terrace carried his argument further by analysing films from other ape language projects and arriving at the same conclusions. Like the turn-of-the-century horse prodigy, Clever Hans, who appeared to do sums in his head and tap out the answers with his hoof, the signing apes appear to be responding at least in part to subtle cues from their trainers.

Reports of Terrace's work have been

widely disseminated. Understandably, they have added to the ape-language controversy, and counter-arguments from other researchers have begun to appear. The author himself, at the end of the volume, explains why he thinks Nim's full linguistic potential may not have been realized, and he outlines a million dollar plan that he believes would produce greater success in a future project.

Language is the central issue, but the book also offers some rare insights into the personality of a bright young chimp. There are some fine photographs, and an informative appendix devoted to sign language. While the definitive work on language in apes has yet to appear, this is certainly both a scholarly and well-written one. □

Jean Berko Gleason is Professor of Psychology at Boston University.

Eclipsing binary light curve analysis

Joel A. Eaton

Language of the Stars: A Discourse on the Theory of the Light Changes of Eclipsing Variables. By Zdeněk Kopal. Pp. 280. (Reidel: 1979.) Hardback Dfl. 120, \$63; paperback Dfl. 55, \$28.95.

THIS book is written for an exceedingly small audience of specialists, or would-be specialists, involved in eclipsing binary light curve analysis. Contrary to the publisher's claim on the back cover, it is not a suitable book for amateur astronomers - other than those with doctorates in theoretical physics. Likewise, it would be inappropriate as a text for a university course about double stars as its content is too narrowly defined, while the references are almost completely restricted to the work of Kopal and his associates.

Throughout the book, Kopal seems to be re-fighting his traditional battle against the Russell model for calculating light curves. In doing this he has for the most part ignored the important work on the synthesis of light curves in the past decade, in which electronic computers have been used to integrate numerically over the visible surfaces of the components of a

model binary system the light received at a distant point. This approach, developed by Leon Lucy, Robert E. Wilson and Graham Hill, to mention but a few of the more important contributors, has practically superseded the Russell model for application to tidally distorted binaries. Yet it rates only a few sentences of disparagement from Kopal.

The subject of this book is a method for determining the geometry of an eclipsing binary star by the Fourier analysis of its light variation, a technique already used extensively by others, Sławek Ruciński for example (*Acta Astronomica* 23, 79; 1973), in studies of the light curves of W Ursae Majoris binaries. The classical method of analysing such light curves is to calculate a theoretical light curve for an assumed set of geometrical and photometric elements, compare these calculations with the observations of brightness as a function of orbital phase, and modify the elements to produce a better fit.

Kopal has devised a scheme for relating the geometrical elements of the model to certain properties of the Fourier transform of the light variation. Specifically, he has derived complicated algebraic equations relating geometrical elements and certain Fourier coefficients. However, after roundly criticizing the Russell model for basing a light curve solution on only three fixed points on the light curve (as was done in the early days), Kopal goes on to base his Fourier-analysis solutions on the power at only four frequencies in the Fourier transform. Indeed, nowhere in the book does he illustrate the Fourier transform of a light curve. □

Inspec have just published *Ion-Solid Interactions: A Comprehensive Bibliography*, compiled by Walter M. Gibson and Henry H. Teitelbaum. The bibliography, which consists of three volumes - *Bibliography*, *Permuted title index* and *Author-title index* - and contains more than 12,000 references, is available from Inspec, Nightingale Road, Hitchin, Herts, UK, price £95, \$195.

Joel A. Eaton is a Research Associate at the Dyer Observatory of Vanderbilt University, Nashville, Tennessee, who specializes in studies of eclipsing binary light curves.

Archaeological chemistry for the curious

Robert Hedges

Archaeological Chemistry. By Z. Goffer. Pp. 376. (Wiley-Interscience: 1980.) £15.20, \$33.

THIS book should appeal most to those wishing for a quick canter around the field of applications of chemistry to archaeology. The first 75 pages give a crash course in chemistry — atoms and molecules in one page, analytical techniques in about 30 — so that, having learnt to sit in the saddle and keep his eyes open, the reader is briskly shown all the major 'sights' of archaeological chemistry.

With research in this area flourishing at present, the scenery is rich indeed. Topics range from ancient cement-making to amino-acid racemization dating; from metal corrosion and its conservation to the identification of dyes used in antiquity. As might be expected, most space is reserved for accounts of ceramics and of metallurgy, but room is also found for such less-common subjects as palaeo-temperature measurement, soil surveys and authentication. Faced with all this, inexperienced readers will be grateful that the treatment of each topic is, perhaps inevitably, superficial. However, they will obtain a clear idea of the extent to which scientific definition can be imposed on the material with which the archaeologist deals, and a notion of some of the surprising results that can follow from the careful use of analytical techniques. References to original literature are frequently cited, although to benefit from them would demand a deeper knowledge than is provided by this book. The style is relaxed, but never unscientific, and the few mathematical hurdles that have not been avoided are taken easily.

Nevertheless, I doubt that the reader will finish the book with any specific knowledge of the kind of research being conducted now, either in terms of individual work or as more synoptic problems (for example, the geographical exploitation of tin during the Bronze Age, the invention of glazing technologies or the dissemination of iron smelting techniques). Nor would I expect an archaeologist reading this book to be much better able to evaluate critically the results of his colleagues' work. For example, in an area where such cooperation is particularly active — the determination of pottery provenance from the comparison of trace element distributions — the subject is treated in just four pages, has no mention of the statistical problems and only a hint of geochemical difficulties.

The subtitle, *A Sourcebook . . .*, makes claims that are not lived up to. Important books and journals are omitted in the bibliography, and comparatively few references are given beyond 1975. To cover such a field with authority would require a much more penetrating book. However, no other single volume attempts

to cover such wide ground, and this one can be recommended to those whose interest is based on curiosity rather than knowledge. □

Robert Hedges is at the Research Laboratory for Archaeology, University of Oxford.

Will we use the ocean wisely?

Henry Charnock

Opportunities and Uses of the Ocean. By D. A. Ross. Pp. 320. (Springer-Verlag: 1980.) DM39.50, \$21.80.

ONCE upon a time and a very good time it was oceanography was the profession of a small number of (almost all) men. They devoted their energy, skill and persistence, and sometimes their money, to exploring, measuring and seeking to understand the complicated structure and motion of the sea. Not, one supposes, in the hope of immediate financial gain; apart from pioneers like Maury and Fitzroy, who realized that an understanding of winds

to the dumping of waste.

Not all of the proposed uses of the ocean are compatible and divergent views are strongly held by a range of interested groups. Dr Ross has set himself the question: "Where is the truth — what are the real problems and opportunities associated with the ocean?" He has attempted "to present some basic information about the ocean in a form that will be understandable to people who are not oceanographers, but who are interested and concerned about the marine environment". So the problems he is concerned with are not so much about the science as about the politics and legalities, while the opportunities are not to understand how the system works but how it can be exploited in a sensible (sustainable?) way.

An objective assessment needs the basic



Energy from under the sea — a natural-gas rig in the North Sea

and currents could shorten sailing-ship passages and improve their owners' profits, most of the early marine scientists seem to have had only the most general notion that knowledge of the sea would prove useful.

Much more effort has been devoted to marine science since the Second World War, and even more money has been poured in since the great powers decided to deploy their nuclear weapons in submarines instead of aircraft. A vast amount of money is now spent in studying the ocean but with changed and more explicit motives — science is increasingly linked with technology and oceanic exploration has given way to exploitation, to the winning of food, minerals and energy and

facts, so he gives 30 pages or so on "how the ocean works". It is difficult to compress such a broad subject into so little space — three pages to physical oceanography, for example — so it is not surprising that some of the explanation seems fluent rather than exact.

With finance as the only criterion, the most important users of the ocean are the navies of the world. Figures are hard to come by but it seems clear that a vast sum is spent each year on a range of activity and equipment mainly concerned with the detection of submarines. Anti-submarine warfare is a multi-billion dollar industry.

So the opacity of the ocean to electromagnetic radiation, which makes it dark, cold and mysterious, has led to a

huge expenditure of scientists' time and taxpayers' money. Fortunately, perhaps, even though the (mainly acoustic) detection systems are complex the modern submarine is relatively safe in the ocean. Ross quotes a comparison between anti-submarine warfare and a game of baseball with the fielders blindfolded. And it is increasingly a game played between only two players, USA versus USSR, both of whom use the ocean as the location of their major weapon systems, their so-called 'second strike' capability. Because both nations have nearly undetectable submarines with frightening nuclear weapons, Ross argues that the opacity of the ocean deters them from starting a nuclear attack — an optimistic view.

If the opacity of sea water is important to submarines its combination of high density and low viscosity is fundamental to merchant shipping; surface ships remain the only practicable way of transporting heavy and bulky cargo over inter-continental distances. Next to military activity the shipping industry spends most on marine affairs (about 10^{11} dollars/year is said to go on building, maintaining and repairing ships). Ships and shipping have changed a great deal in recent decades: passenger liners are no more and cargo ships have become much more specialized, especially as regards the handling and stowage of their loads. They now need far smaller crews and in spite of much improved navigational aids collisions and other accidents still happen. Such catastrophes may lead to acute pollution of the *Torrey Canyon* type and one sympathizes with Ross's advocacy of "clear and positive control of ships, especially near or within coastal waters". But a comparison of aircraft and marine traffic control shows that this will need significant changes in the education and attitude of seafarers: this is a sociological problem which badly needs study.

The resources of the ocean with which most people are familiar are animal and mineral (it seems unlikely that the vegetable phytoplankton will be harvested in bulk, though seaweed is an important product and its cultivation a new and growing industry). There is much argument about the total amount of fish and related creatures that could be safely harvested — perhaps 2×10^8 tons/year is an optimistic figure. But it seems clear that marine biological resources are not the answer to the basic food problem posed by half the world's population being short of protein. Poorly understood and rather ineffectively controlled, fishing is perhaps the classical example of a non-profit industry. There may be an opportunity to exploit hitherto untaken species (krill is the obvious example); one can improve processing so as to convert trash fish into palatable products; one can develop and improve aquaculture techniques. All these will help supplement the world's protein requirements but seem unlikely to make a

Soil sugars for the soil scientist

M. Schnitzer

Nature and Origin of Carbohydrates in Soils. By M. V. Cheshire. Pp. 216. (Academic: 1980.) £15.80, \$36.50.

ABOUT 10% of the organic matter in soils occurs as carbohydrates — mainly in the form of polysaccharides comprised of at least seven neutral, two acidic and two basic sugars — which originate from plant and animal remains and from microbial syntheses. From an agricultural point of view, their most important functions are to supply microorganisms with readily available food and to bind soil particles into stable aggregates which improve soil aeration, root development and ease of cultivation, and prevent erosion.

The first four chapters of this book deal with the composition and analysis of carbohydrates and polysaccharides in soils. Especially useful are recommended procedures for the hydrolysis and analysis of freed sugars, uronic acids and amino sugars by colorimetric, gas chromatographic and enzymatic methods. A

major contribution.

Mineral, as distinct from biological, marine resources provide a clearer problem. Most are not renewable so the problem is to find them, extract them and process them, without harming the environment. Here Dr Ross is on home ground, giving a clear account of the probability of exploiting the sediments of the Red Sea brine pools and of the possibility of using the better known manganese nodules. In shallower water offshore hydrocarbons are already a major industry, and will be for decades. Their yearly value (about 4×10^8 dollars) already exceeds that of fisheries.

Marine pollution is a diffuse and ill-defined subject. There is a great lack of information on sources, cycles and sinks of chemicals in the sea. Much of the most objectionable material comes from industrial sources, mainly in developed countries (the degree of marine pollution correlates closely with gross national product). So it would be possible, at a price, to control it at source. This is one of the points made strongly by the developing nations at the UN Conference on the Law of the Sea, and one can readily sympathize with their point of view. Not much interested in the military and shipping aspects, they are concerned to ensure that the industrialized nations do not gain a disproportionately large share of the (perhaps over-stated) marine resources. The scientific study of the ocean comes relatively low on their list of priorities (after agriculture, roads, hospitals, literacy and such) so they see little harm in the pace of

problem in such analyses is that polysaccharides in soils appear to be chemically linked to proteins and humic materials; so far separation from humic materials has been more successful than from proteins.

The next section is concerned with the metabolism of carbohydrates in soils. Incubation experiments with ^{14}C -labelled glucose show that while microorganisms can synthesize soil sugars, the overall composition of microbial polysaccharides differs from that of soil polysaccharides as a whole and can only account for a part of it. It is estimated that the live biomass constitutes only 2.5% of the weight of soil carbohydrates.

The author has made a very useful contribution by bringing together the essence of widely scattered literature published over a period of 85 years. To the best of my knowledge this is the first book to deal with carbohydrates in soil; its appearance will be welcomed by those interested in the chemistry, biology and management of soil organic matter. □

M. Schnitzer is Program Leader of soil chemistry and biology research at the Chemistry and Biology Research Institute, Agriculture Canada, Ottawa.

marine research being slowed down. Even the so-called developed nations prefer to gain control over as much of the ocean and seabed as possible rather than to make it free for oceanographers to study the basic phenomena.

Ross ends with a chapter "Innovative Uses of the Ocean", including energy from the sea; climate and the ocean; fresh water from the sea; drugs from the sea; disposal of nuclear waste; research on satellite oceanography; the prospects for offshore islands. Again the chapter is too short to do justice to the variety of topics and the author has not been able to polish his writing sufficiently. Altogether the book, though superficially attractive, gives the impression that it would have been much improved by more attention to detail, both in text and illustration. The photographs are good but some of the map projections unfortunate; the text has confusing expressions and rather a lot of minor misprints, even in the prefatory poem (*The Sea and the Hills*, from Kipling's *The Five Nations* — and a good example of his sound and fury style it is).

No one could possibly disapprove of the author's motives — it would be marvellous if politicians and lawyers and admirals and (perhaps especially) multi-national moguls knew and cared more about the sea. But I fear it will need more than a book like this. L

Henry Charnock is Professor of Physical Oceanography at the University of Southampton.

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Mathematics

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- EGGINTON, J. *The Poisoning of Michigan*. Pp. 351. ISBN 0-393-01347-2. (W. W. Norton: 1980.) \$13.95.
- FORTESCUE, J. A. C. *Environmental Geochemistry: A Holistic Approach*. Pp. 237. ISBN 3-540-90454-9. (Springer-Verlag: 1980.) DM 69, \$40.80.
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- POMEROL, C. (ed.). *Geology of France with twelve itineraries and a geological map at 1:2,500,000*. Guides Géologiques Régionales. Pp. 256. Pbk ISBN 2-225-67001-3. (Masson: 1980.) Np.
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- DROOGERS, A. The Dangerous Journey. Symbolic Aspects of Boys' Initiation among the Wagena of Kisangani, Zaire. Change and Continuity in Africa. Pp.416. Flexi ISBN 90-279-3357-X. (Global Book Resources: London, 1980.) £15.75.
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- IASZLO, E. *et al.* The Obstacle to the New International Economic Order. Pergamon Policy Studies on the New International Economic Order. Pp. 144. Hbk ISBN 0-08-025110-2, flexi ISBN 0-08-025110-2. (Pergamon: 1980) Hbk £8.75; flexi £3.55.
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- MATURANA, H. R. and VARELA, F. J. Autopoiesis and Cognition. The Realization of the Living. Pp. 140. Hbk ISBN 90-277-1015-5; pbk ISBN 90-277-1016-3. (Reidel: 1980.) Hbk Dfl. 55, \$28.95; pbk Dfl. 27.50, \$14.50.

ANNOUNCEMENTS

Awards

Three Royal Medals, known also as The Queen's Gold Medals, of the Royal Society are to be awarded to: **Sir Denys Wilkinson** (Vice Chancellor of the University of Sussex); **Professor Henry Harris** (Regius Professor of Medicine in the University of Oxford); **Dr J. P. Wild** (chairman of CSIRO).

The Council of the Royal Society has made the following award of medals: The Copley Medal to **Sir Derek Barton** (L'Institut de Chimie des Substances Naturelles, Gif-sur-Yvette France); The Rumford Medal to **Professor W. F. Vinen** (University of Birmingham); The Davy Medal to **Professor A. W. Johnson** (University of Sussex); The Darwin Medal to **Professor S. Wright** (University of Wisconsin); The Hughes Medal to **Dr F. J. M. Farley** (Royal Military College of Science, Shrivenham); The Mullard Prize and Medal to **Sir Edward Abraham** (University of Oxford). The Council has awarded the Royal Society Wellcome Foundation Prize for 1980 to **Dr C. Milstein** (University of Cambridge).

The 1980 Paul-Martini-Prize has been divided between **Dr Winfried Günselmann** (Institut für Medizinische Dokumentation und Statistik der Universität Erlangen-Nürnberg) and **Professor David Grahame-Smith**, **Dr Jeffrey Aronson** and **Dr Alan Ford** (University of Oxford).

The recipients of the Wolf prizes — 1980 are: in agriculture, **Professor Karl Maramorosch** (Rutgers University, New Jersey); in mathematics, **Professor Emeritus Henri Cartan** (Université de Paris) and **Professor Andrei N. Kolmogorov** (Moscow State University); in chemistry, **Professor Henry Eyring** (University of Utah); in physics, **Professor Michael E. Fisher** (Cornell University, Ithaca), **Professor Leo P. Kadanoff** (Brown University, Providence) and **Professor Kenneth G. Wilson** (Cornell University, Ithaca); in medicine, **Dr César Milstein** (Medical Research Council), **Professor Leo Sachs** (The Weizmann Institute of Science) and **Dr James L. Gowans** (Medical Research Council).

The Wellcome Trust have awarded the following Senior Lecturerships in medical and veterinary science: **Dr J. K. Aronson** (University of Oxford); **Dr B. Burchell** (University of Dundee); **Dr M. J. Doenhoff** (London School of Hygiene & Tropical Medicine); **Dr S. E. File** (University of London); **Dr S. L. Lightman** (St Mary's Hospital Medical School); **Dr S. Tomlinson** (University of Sheffield); **Dr R. D. Ward**, (Liverpool School of Tropical Medicine); **Dr I. K. Welsh**, (Royal Postgraduate Medical School).

The next prize of the Foundation de Physiopathologie Professeur Lucien Dautrebande for work on human or animal clinical physiopathology, will be awarded during 1982. Further information from: The Office of the Foundation, 35, chaussée de Liège à 5200 Huy, Belgium.

Appointment

Alan R. Katritzky, former Professor of Chemistry in the University of East Anglia, has been appointed from 1 August, to the new Kenan Chair of Organic Chemistry at the University of Florida, Gainesville.

Meetings

20-21 October **Waste Utilization**, Chicago (S. A. Bortz, ITT Research Institute, 10 West 35th St, Chicago, Illinois 60616).

27 October, **Enzyme Mechanisms and the Design of Enzyme Inhibitors**, London (Conference Secretariat, Society of Chemical Industry, 14 Belgrave Square, London SW1, UK).

27-28 October, **Managing Periodicals Profitably**, London (Benn Business Promotions Ltd, Press House, 25 High St, Edenbridge, Kent, UK).

28 October, **The Resources and Resourcefulness of Agriculture**, London (PA to the Director, The Ciba Foundation, 41 Portland Place, London W1, UK).

27-31 October, **1980 Photovoltaic Solar Energy Conference**, Cannes (Mr D. Nicolay, Commission of the European Communities, DGXIII A, Kirchberg, Luxembourg).

29 October, **Food Science and Technology in Developing Countries**, Glasgow (Institute of Food Science and Technology, 105-111 Euston St, London NW1, UK).

29-30 October, **The Manipulation of Genetic Systems in Plant Breeding**, London. (The Royal Society, 6 Carlton House Terrace, London SW1, UK).

2-5 November, **Applications of Accelerators in Research and Industry**, Texas (Conference on Application of Accelerators, PO Box 5368, NTSU Station, Denton, Texas 76203).

4-5 November, **Planetary Exploration**, London (The Royal Society, 6 Carlton House Terrace, London SW1, UK).

6 November, **Is It a Virus?** London (The Royal Society, 6 Carlton House Terrace, London SW1, UK).

7 November, **Nutrition and Diabetes**, Edinburgh (Dr M. I. Gurr, Shinfield, Reading, UK).

11-13 November, **The Food Industry and its Effluents: Treatment and Conservation**, Norwich (Mrs H. Gibbons, AHS, Owles Hall, Buntingford, Herts, UK).

12-13 November, **The Perception and Assessment of Risk**, London (The Royal Society, 6 Carlton House Terrace, London SW1, UK).

20-21 November, **Interaction of the Platelets and the Vessel Wall**, London (The Royal Society, 6 Carlton House Terrace, London SW1, UK).

24 November, **The Physics of Microwave Devices**, London (The Meetings Officer, 47 Belgrave Square, London SW1, UK).

24-28 November, **Combination Processes in Food Irradiation**, Colombo (PO Box 100, Vienna International centre, A-1400 Vienna, Austria).

25 November, **Memory Its Function, Technology and Impact**, London (The Royal Society, 6 Carlton House Terrace, London SW1, UK).

26 November, **Instrumentation and Analysis — Remember the Burette?**, London (Society of Chemical Industry, 14 Belgrave Square, London SW1, UK).

27-28 November, **Gamma ray Astronomy**, London (The Royal Society, 6 Carlton House Terrace, London SW1, UK).

10-12 November, **Automated Instrumentation for the Analytical laboratory**, Central New Jersey (The Centre for Professional Advancement, East Brunswick, New Jersey 08816).

17-19 November, **Practical Mass Spectrometry**, Central New Jersey (The Centre for Professional Advancement, East Brunswick, New Jersey 08816).

2-4 December, **Offshore Environment in the 80s**, St John's Newfoundland (Information and Public Relations Officer, Killam Library, Dalhousie University, Nova Scotia B3H 4HB).

3-5 December, **Practical Colorimetric Chemical Analytical Methodology**, Central New Jersey (The Centre for Professional Advancement, East Brunswick, New Jersey 08816).

9-12 December, **Industrial Membrane Technology**, Central New Jersey (The Center for Professional Advancement, East Brunswick, New Jersey 08816).

15-17 December, **Applied Absorption Technology**, Central New Jersey, The Center for professional Advancement, East Brunswick, New Jersey 08816).

15-19 December, **Genetic Engineering**, Washington (Battelle, Memorial Institute, 505 King Avenue, Columbus, Ohio 43201).

7-9 January, **Plastics Additives**, Central New Jersey (The Center for Professional Advancement, East Brunswick, New Jersey 08816).

27 January, **New Forms of Silicon for Use in Semiconductor Devices**, London (The Meetings Officer, The Institute of Physics, 47 Belgrave Square, London SW1, UK).

3-6 February, **Industrial Membrane Technology**, San Francisco (The Centre for Professional Advancement, East Brunswick, New Jersey 08816).

8-15 February, **Immunoglobulin Idiotypes and their Expression**, Salt Lake City (ICN — UCLA Symposia, Molecular Biology Institute, University of California, Los Angeles, California 90024).

15-20 February, **Differentiation and Function of Hematopoietic Cell Surfaces**, Keystone (ICN — UCLA Symposia, Molecular Biology Institute, University of California, Los Angeles, California 90024).

22 February — 1 March, **Mechanisms of Chemical Carcinogenesis**, Keystone (ICN — UCLA Symposia, Molecular Biology Institute, University of California, Los Angeles, California 90024).

1-8 March, **Cellular Recognition**, Keystone (ICN — UCLA Symposia, Molecular Biology Institute, University of California, Los Angeles, California 90024).

8-12 March, **Genetic Variation among Influenza Viruses**, Salt Lake City (ICN — UCLA Symposia, Molecular Biology Institute, University of California, Los Angeles, California 90024).

9-13 March, **Structure and DNA Protein Interactions of Replication Origins**, Keystone (ICN — UCLA Symposia, Molecular Biology Institute, University of California, Los Angeles, California 90024).

15-20 March, **Development Biology using Purified Genes**, Keystone (ICN — UCLA Symposia, Molecular Biology Institute, University of California, Los Angeles, California 90024).

30 March-3 April, **The Theory and Practice of Solid Dosage Form Manufacture**, London (Mr R. E. Marshall, Dept of Pharmaceutical Sciences, The Pharmaceutical Society of Great Britain, 1 Lambeth High St, London SE1, UK).

30 March-2 April, **Viral Hepatitis**, New York (New York University Postgraduate Medical School, 550 First Ave, New York, New York 10016).

5-11 April, **The Sea in the Service of Man**, Monaco (International Bureau, 16 Regency St, Westminster, London SW1, UK).

5-10 April, **2nd European Congress of Biotechnology**, Eastbourne (ECB2 Secretariat, Society of Chemical Industry, 14 Belgrave Square, London SW1, UK).

6-10 April, **Time and Space in the Emergence of the Atmosphere**, London (R. W. Simms, Dept of Zoology, British Museum (Natural History), Cromwell Road, London SW7, UK).

7-9 April, **Assessment of Imaging Systems**, Reading (Sira Institute Ltd, South Hill, Chislehurst, Kent, UK).

7-10 April, **Properties of Oriented Polymers**, Leeds (The Institute of Physics, 47 Belgrave Square, London SW1, UK).

8-10 April, **Spring Meeting — Association of Radiation Research**, Dundee (Dr I. V. Chapman, Dept of Medical Biophysics, University of Dundee, UK).

13-16 April, **Electroanalysis in Clinical, Environmental and Pharmaceutical Chemistry**, Cardiff ((Short Courses Section Electroanalysis Symposium), UWIST, Cardiff, UK).

29 April-1 May, **Energy Storage**, Brighton (BHRA Fluid Engineering, Cranfield, Bedford, UK).

2 June-3 July, **Advanced Training of Foreign Participants in Remote Sensing — Geologic Interpretation**, Flagstaff (John A. Reinemund, US Geological Survey, National Centre — M/S 917, 12201 Sunrise Valley Drive, Reston, Virginia 22092).

7-12 June, **International Leucocyte Culture Conference**, Heidelberg (Dr H. Kirchner, Institut für Virusforschung, DKFZ Im Neuenheimer Feld 280, D-6900 Heidelberg, FRG).

22-26 June, **Affinity, Chromatography and Related Techniques**, Nijmegen (Dr T. C. J. Gribnau, Katholieke Universiteit, Nijmegen, The Netherlands).

22-27 June, **3rd European Symposium for Stereology**, Ljubljana (Prof. Dr M. Kalisnik, Institute for Histology and Embryology, PO Box 10, 61105 Ljubljana, Yugoslavia).

19-24 July, **Chemotherapy**, Florence (Secretariat, 12th International Congress of Chemotherapy, Via della Scala, 10, Florence, Italy).

21-30 July, **General Assembly of IASPEI**, Ontario (Prof. A. E. Beck, Dept of Geophysics, University of Western Ontario, London, Ontario, Canada N6A 5B7).

11-12 August, **Open Symposium on Remote Sensing**, Washington (Dr D. L. Croom, Rutherford and Appleton Laboratories, Ditton Park, Slough, Berks, UK).

17-22 August, **Earth Tides**, New York (J. T. Kuo, Columbia University, New York, 10027).

23-28 August, **Solar World Forum**, Brighton (UK ISES, 19 Albemarle St, London W1, UK).

23-29 August, **7th International Biophysics Congress and 3rd Pan-American Biochemistry Congress**, Mexico City (Cerrada de Popocatepetl, México 13 D.F. Mexico).

31 August-3 September, **Computer Applications in Fermentation Technology**, Manchester (Society of Chemical Industry, 14 Belgrave Square, London SW1, UK).

9-11 September, **Radiopharmacology**, Chicago (Dr L. G. Colombetti, Pharmacology Dept, Loyola University, Stritch School of Medicine, Maywood Illinois 60153).

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